Exploring the Transcriptional Influence of Upstream Elements on a Human U6 Small Nuclear RNA Gene Promoter

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ABSTRACT

EXPLORING THE TRANSCRIPTIONAL INFLUENCE OF UPSTREAM ELEMENTS ON A HUMAN U6 SMALL NUCLEAR RNA GENE PROMOTER. *John D. Hixson (Dr. Gary R. Kunkel), Department of Biochemistry and Biophysics, Texas A&M University.*

Past studies have shown that the promoter of a human U6 small nuclear RNA gene contains two distal elements that increase the transcriptional level of the gene. One of these elements, termed OCT, is an octamer motif that binds the recombinant Oct-1 transcription factor. In addition, another sequence element, termed NONOCT, is located nearby and also appears to stimulate transcription in conjunction with the Oct element. This NONOCT element does not bind the Oct-1 transcription factor, but rather another transcription factor found in crude cellular extracts. Using the wildtype promoter containing both the OCT and NONOCT elements, transfection and in vitro transcription experiments have demonstrated an increased level of transcriptional activation of the U6 snRNA promoter.

In an effort to further characterize the influence of these elements, plasmid constructs containing the U6 snRNA proximal promoter and a cytidine-free oligonucleotide reporter gene were used in transcriptional assays. The individual OCT and NONOCT elements were ligated immediately upstream of the U6 proximal promoter into separate plasmids, as was a combined OCT/NONOCT element. Selected plasmids were then utilized for in vivo transfection and in vitro transcription assays to determine the effects of the individual elements, their orientation, and their dosage on the transcriptional activation of the promoter. The presence of both of these elements results in an increased level of transcriptional activity by the U6 snRNA promoter over the promoter that does

not possess either of these elements. Furthermore, the OCT element has a greater effect on the transcriptional level than does the NONOCT element. A promoter with both elements combined, however, shows slightly more activation of the promoter than the OCT element alone, indicating an additive effect of the influences of both elements. However, there appears to be no additional effects related to the dosage or orientation of these upstream elements.

INTRODUCTION

Human U6 small nuclear RNA (snRNA) genes are a class of RNA polymerase III-transcribed genes containing upstream promoters that are repeated many times within the human genome (1,2). Many small nuclear RNAs are utilized in processing other RNA transcripts before translation into functional proteins. The U6 snRNA is an integral part of a protein-RNA complex known as a spliceosome, which removes introns from RNA transcripts, forming a messenger RNA that can be translated into a protein (3). Thus, U6 snRNA plays an integral role in the central process of genetics, that is, the conversion of the genetic code into functional proteins.

Studying the mechanism of gene transcription in eukaryotes is quite interesting because there are several different types of polymerases that are used in the transcription of different gene classes. RNA polymerase II is used in the transcription of protein-coding genes and some snRNA genes similar to the U6 snRNA gene. For example, RNA polymerase II transcribes the U1 and U2 snRNA genes which have a related RNA processing function to the U6 snRNA. RNA polymerase III, on the other hand, is utilized in the transcription of transfer RNA genes and other snRNA genes, such as those encoding U6 snRNA. Historically, the promoter structures of RNA polymerase II- and III-transcribed genes were thought to be very different. Generally, the polymerase II promoter is located in the 5' flanking region, while the polymerase III promoter is located downstream of the start site of transcription. However, the promoter regions of both polymerase II and III-transcribed snRNA genes are very similar in structure, making them excellent comparative models to study the mechanism of RNA polymerase transcription.

The polymerase III U6 promoter appears to be more complex than the

polymerase II snRNA gene promoter. Within 70 base pairs of the transcription start site, a polymerase III promoter contains a proximal sequence element (PSE) and a T-A nucleotide-rich element called the TATA element. In addition, polymerase III promoters contain an enhancer-like octamer motif in the distal region 200 base pairs upstream (4). This type of RNA polymerase III promoter structure is representative of the human U6 snRNA gene utilized in this study (1,2). The promoter region of vertebrate polymerase II snRNA genes also possesses the proximal sequence element (PSE) and the distal octamer motif, but does not possess the TATA element found in polymerase III genes (5) (Figure 1).

Because snRNA gene transcription must be initiated often in order to produce abundant snRNAs, both the polymerase II and III promoters must be efficient. In fact, the distal region containing the octamer enhancer-like motif has been shown to cause a five to one-hundred fold increase in the transcriptional activity of both polymerase II and III promoters in transfected mammalian cells or *Xenopus* oocytes (6,7,8,9,10). In addition to the enhancer-like octamer motif, both polymerase II and III promoters have been shown to possess a second sequence element in the same region that may also act in an enhancer-like fashion. Among the many polymerase II and III-transcribed genes, however, there are many different forms of this second sequence element; only the octamer motif is conserved for each of these promoters (11,12,13,14,15,16).

As expected for RNA polymerase III-transcribed genes, the human U6 snRNA promoter appears to be activated by these distal elements, the conserved octamer motif (OCT) and a second sequence element that has been termed NONOCT (17). The distal octamer motif has been shown to bind to the Oct-1 transcription factor, further demonstrating the influence of the OCT element on the polymerase III promoter (9,10,17). Furthermore, the addition of this Oct-1 transcription factor increases polymerase III-mediated transcription in vitro

(17,18,19). In the U6 snRNA promoter, the second distal element, NONOCT, does not bind this Oct-1 transcription factor but does bind another protein in a crude cellular extract (17). Based on both polymerase II and III promoter studies, the presence of the conserved OCT element acts an important transcription activator in vivo (17,19,20). In the U6 snRNA promoter specifically, the distal region containing both the OCT and NONOCT elements substantially increases the transcriptional level of the gene by polymerase III (17). Figure 2 presents a representation of the complete U6 snRNA promoter, including the proximal PSE and TATA elements and the distal OCT and NONOCT elements. The full activation of the U6 snRNA promoter is directly dependent on the presence of both these OCT and NONOCT elements (17).

This study continues the characterization of these two distal elements by observing the effects of orientation and dosage on the transcriptional activation of the U6 snRNA promoter. In addition, the OCT and NONOCT elements will be studied both individually and in combination using an oligonucleotide termed NPLUSO. These studies will ultimately be used to help understand the specific mechanism of snRNA gene activation and can be applied to a wide range of other studies involving RNA polymerase II and III-transcribed genes.

MATERIALS AND METHODS

Plasmid Constructions: Experimental plasmids were first constructed containing a human U6 wildtype promoter with the TATA and PSE proximal elements and a reporter cytidine-free element (CFREE) attached to the U6 promoter. Previously, a plasmid was constructed containing the U6 promoter attached to the CFREE reporter element (U6/CFREE) (21). The 5'-flanking region of the promoter was deleted to position -84 by restriction with DraI and EcoRI and removed from the plasmid. Additionally, a pGEM3zf(-) plasmid (Promega) was restricted with SmaI and EcoRI. Each of these digests was run through a 1% agarose gel to separate the U6/CFREE and cut pGEM3zf(-) fragments; these fragments were then electroluted in 0.5X TBE to isolate them from agarose. The electroluted fragments were then fractionated over a Sephadex CL-4B column and again run through a 1% agarose gel to analyze the concentration of both the U6/CFREE and cut pGEM3zf(-) in buffer. The dl-84/U6/CFREE element was then ligated into the pGEM3zf(-) cut with SmaI and EcoRI to form the complete plasmid dl-84/U6/CFREE with no upstream distal elements (17). This plasmid was termed dl-84 and contains neither of the enhancer-like elements OCT and NONOCT. This ligated plasmid was then used to transform E. coli-HB101 competent cells. Plasmid DNA from the transformations was screened by restriction enzyme analysis, and the presence of the dl-84/U6/CFREE insert was verified by dideoxy sequencing.

Plasmids containing the OCT, NONOCT, and NPLUSO oligonucleotides were constructed by restricting the dl-84/U6/CFREE plasmid with BamHI and ligating the oligonucleotides into this site. The oligonucleotide sequences used were as follows ("top" strand; each strand contained GATC overhangs): OCT (GATCCATATTTGCATAT), NONOCT (GATCAGGGCCTATTTCCCAT

GATTCCTTCA), and NPLUSO (GATCCTATTTCCCATGATTCCTTCAT ATTTGCATAT). The ligation reactions for each oligonucleotide were then used to transform HB101 competent cells and which were grown on LB/Amp plates at 37°C. The resulting colonies were randomly selected and cultured overnight. Eight colonies for each oligonucleotide were chosen in order to get a random sampling of different numbers and orientations of each oligonucleotide in the plasmid. Each of these plasmid minipreps was digested with MspI in order to determine which plasmids actually contained distal oligonucleotides. DNA fragments were then electrophoresed on a 1% agarose gel to determine the size of the plasmid insert and thus the presence of the OCT, NONOCT, or NPLUSO elements. As an example, Figure 3 shows the gel electrophoresis results for several of the OCT plasmids. In the plasmids that contain inserts, the fourth-slowest band created by restriction digest with MspI migrates slower than the same band seen in plasmid without an insert. This indicates a larger fragment containing single or multiple copies of the OCT insert. In Figure 3, lanes 1, 3, and 4 indicate the presence of inserts, while lane 2 represent a restriction map for a plasmid that does not contain an insert. For the NONOCT and NPLUSO ligations, colony lifts were performed on the LB/Amp plates using nylon filters, followed by the hybridization of these filters with radiolabelled NPLUSO oligonucleotide. From the results of these hybridization reactions, eight positive colonies were selected and analyzed as above for the OCT plasmids.

The distal oligonucleotide inserts for each of positive plasmids were checked by dideoxy sequencing to determine the number and orientation of the OCT, NONOCT, and NPLUSO elements. Based on these sequencing reactions, the following plasmids were successfully constructed: OCT4 containing one forward copy and one reverse copy of the OCT element, OCT5 containing four reverse copies of the OCT element, OCT7 containing one reverse copy of the

OCT element; NONOCT10 containing one forward copy of the NONOCT element, NONOCT13 containing two forward copies of the NONOCT element; NPLUSO10 (P10) containing one reverse copy of the NPLUSO element, NPLUSO13 (P13) containing one forward copy of the NPLUSO element not connected to the dl-84 promoter element, and NPLUSO14 containing four reverse copies of the NPLUSO element. Table 1 lists each of these constructed plasmids with the oligonucleotide sequences.

Purified Plasmid Preparations: Large scale, purified preparations of each of the sample plasmids were performed by alkaline lysis of bacterial cells, followed by cesium chloride gradient centrifugation. The plasmid DNA concentrations of these preparations were determined by absorbance at 260 nm and verified by ethidium bromide-stained agarose gels.

Transfection Experiments: Transfection experiments were performed for each of the plasmids in order to determine the effects of the upstream elements on the level of promoter activity under in vivo conditions. A transfection cell line of 293 cells was first grown in DMEM media with 10% fetal calf serum and incubated continuously at 37°C in a 5% carbon dioxide atmosphere. This line of 293 cells was then cultured in 100-mm dishes, and the cells were cotransfected with 10 μg of the plasmid DNA samples and 5 μg of a control plasmid, pGEM cβ3, containing a chicken beta-tubulin gene. This control plasmid served as an internal control to normalize for the efficiency of the transfections and the RNA recovery. The transfection experiments were performed using the calcium phosphate-mediated procedures previously outlined (22,23). The total RNA from

each plate was isolated after approximately 48 hours of incubation. The RNA isolation was accomplished by initially scraping the cells from the plate and washing with phosphate-buffered saline. The cells were then pelleted and dissolved in a solution containing 8M guanidine-HCL, 0.3M sodium acetate, 1% sarkosyl, and 1% β-mercaptoethanol. This solution was homogenized by multiple passages through a 21-gauge needle and cleared by centrifugation at 14K RPM for 30 minutes in a microfuge; the nucleic acids were then precipitated in ethanol. The precipitate was pelleted and then resuspended in a solution containing 4M guanidine-isothiocyanate and 25 mM sodium citrate (pH 7.0). The nucleic acids were precipitated in ethanol, pelleted, and consecutively washed several times in 95% and 70% ethanol. The washed pellets were resuspended in 20 µl deionized water and then heated at 65°C for 15 minutes.

These RNA preparations were then used to quantitate the amount of RNA produced by the U6 promoter in each of the experimental plasmids. Specific transcripts of the CFREE reporter (U6 promoter) and the beta-tubulin control were detected by primer extension with CFREE and beta-tubulin (BT-68) radiolabelled primers, respectively. For purification, the CFREE oligonucleotide was size-fractionated by electrophoresis, eluted from the gel slice, and chromatographed through a Sephadex G25 column. The BT-68 oligonucleotide had been previously purified by the same procedure. Each oligonucleotide was phosphorylated using gamma-^{32}P-ATP and T4 polynucleotide kinase, followed by a hybridization reaction to anneal these radiolabelled CFREE and BT-68 primers to the specific RNA transcripts. The experiment was completed by extending the primer using reverse transcriptase to form a complete, end-labelled DNA fragment. These labelled fragments were then electrophoresed on a 10% polyacrylamide denaturing gel. The relative amount of transcript produced by each sample plasmid was quantitated by comparing the radioactivity found in the

specific bands for CFREE after background subtraction and normalization to the BT-68 signal. This relative level of radioactivity was directly quantitated using a Fujix BAS2000 Phosphoimager (Fuji).

In Vitro Transcription Experiments: In vitro transcription experiments were performed in order to determine the effects of the upstream elements on the U6 promoter under in vitro conditions. To perform the CFREE transcription reactions, 200 ng of each sample DNA plasmid and 300 ng of pGEM3zf(-) plasmid were mixed in a solution containing 2 μg/μl alpha-amanitin, 2 mM dithiothreitol, 2 mM magnesium chloride, 0.5 mM ATP, 0.5 mM UTP, 0.025 mM GTP (unlabelled), 8 mM HEPES, 8% glycerol, 40 mM potassium chloride, 0.08 mM EDTA, and 10 μCi α-^{32}P-GTP. In addition, each reaction contained 100 μg of protein from a HeLa cell S100 extract (24). This mixture was then incubated at 30°C for one hour. Following this incubation, the reaction (25 μl volume) was immediately stopped by adding a solution (175 μl volume) containing 2.3 M ammonium acetate, 0.1% sodium dodecyl sulfate, 5 μg yeast tRNA, and 3.3 M urea. In addition, a radiolabelled synthetic transcript previously prepared (24) was added in order to serve as a recovery control for the sample RNA transcripts. The RNA transcripts were separated on a 10% polyacrylamide-8.3 M urea minigel, and amounts were quantitated using the Fujix BAS2000 Phophoimager (Fuji).

RESULTS

Several plasmids containing various combinations of the OCT, NONOCT, and NPLUSO elements were successfully constructed. In order to best study the effects of each of the OCT and NONOCT distal elements, it was first necessary to construct experimental plasmids that would test the direct effects of these elements on the U6 snRNA promoter. The goal was to generate plasmids containing each of the three elements in various orientations and combinations. This included the individual OCT and NONOCT elements and the combined NPLUSO element in separate plasmids. In addition, plasmids containing multiple copies of each of these elements were created in order to study the dosage effect on the activation of the U6 snRNA promoter. These plasmid constructions were performed by ligating each of the OCT, NONOCT, and NPLUSO elements into the upstream region of a dl-84/U6/CFREE plasmid. Candidate subclones were chosen randomly, and plasmid DNAs were restricted with MspI to check for the presence of a sizable insert. From this sampling, several different plasmids were created, as verified by dideoxy sequencing of the inserts. Although not all combinations of the OCT, NONOCT, and NPLUSO elements were obtained, a substantial sampling was successfully produced. Table 1 provides information on the plasmids created and the inserts that they contain.

In vivo transfection and in vitro transcription assays both demonstrate increased transcriptional activity due to the presence of the distal upstream region. The initial transfection assay was used to compare the activity of the U6/CFREE (containing ~470 bp of wildtype U6 upstream sequence) and dl-84/U6/CFREE plasmids, along with the other experimentally-prepared plasmids. Although the results of the this assay indicated a higher level of transcription for the U6/CFREE plasmid, the beta-

tubulin control band was not detected. Subsequent tests showed that the CFREE and control beta-tubulin oligonucleotide radiolabelling was inefficient. After relabelling the CFREE and BT-68 oligonucleotides, the CFREE RNA isolated from the original transfection assay was hybridized and lengthened by reverse transcriptase. After separating the primer extension products through a 10% polyacrylamide denaturing gel, the results again indicated that the U6/CFREE plasmid supported a higher level of transcription than the dl-84/U6/CFREE plasmid. The calcium phosphate-mediated transfection assay was completed once more, again using the full complement of experimental plasmids. In addition, two sets of hybridization and reverse transcriptase reactions were performed using the RNA isolated from this set of transfection assays. Both of the polyacrylamide gels of these reactions also indicated higher levels of transcriptional activity for the U6/CFREE plasmid than for the dl-84/U6/CFREE plasmid. Figure 4 shows an autoradiograph from one of these polyacrylamide gels, indicating that the transcriptional activity of the U6/CFREE plasmid (lane 1) is higher than that of the dl-84/U6/CFREE plasmid (lane 2). These results indicate that the upstream region of the the U6 snRNA promoter, containing the OCT and NONOCT elements, is required for the most efficient transcriptional activity of the promoter. These findings substantiate previous work which also used the results of transfection experiments to determine the effects of upstream elements on the U6 snRNA promoter (6,17).

In addition to in vivo transfection assays, in vitro transcription assays of the U6/CFREE and dl-84/U6/CFREE plasmids were used to compare transcriptional activation levels. In vitro transcription assays were performed for these two plasmids using a crude HeLa S100 whole cell extract. As seen in Figure 5, this transcription experiment indicated a higher level of transcription for the U6/CFREE plasmid containing the OCT and NONOCT elements (lane 2)

than for the dl-84/U6/CFREE plasmid with no upstream elements (lane 1). These differences in transcriptional activity have not been previously shown using in vitro transcription assay techniques. However, these results clearly indicate the activating influence of the upstream region on the U6 snRNA promoter.

In vivo transfection and in vitro transcription assays indicate that the constructed dl-84/U6/CFREE plasmids containing various OCT, NONOCT, and NPLUSO elements have a higher transcriptional activity than both the wildtype U6/CFREE and dl-84/U6/CFREE control plasmids. In both sets of transfection assays using all of the experimental plasmids, quantitation of the primer extension products indicated that the presence of OCT, NONOCT, and NPLUSO inserts increased the transcriptional activity greater than that of the U6/CFREE plasmid. This general trend can be observed in Figures 6 and 7, which are autoradiographs of the gel-separated primer extension products from two separate transfection assays. RNA recovery and subsequent quantitation was a problem for several of the samples (Figure 6, lane 7 (OCT 7); Figure 6, lane 1 (P10); and Figure 7, lane 7 (OCT 7)). Although these differences in recovery greatly affect the observations of the autoradiograph results, normalization to the BT-68 control demonstrate the trend in transcriptional activity. In Figure 6, the OCT (lanes 5 and 6) and the NONOCT (lanes 9 and 10) plasmids showed a high level of transcriptional activity compared to the dl-84/U6/CFREE plasmid (lane 8). The quantitative normalization of this activity shown in Figure 8 also indicates that the OCT and NONOCT plasmids are slightly more activating than the U6/CFREE wildtype plasmid. In Figure 7, the NPLUSO (lane 4) plasmid also shows a high level of transcriptional activity compared to the dl-84/U6/CFREE plasmid (lane 8). Furthermore, the normalized results in Figure 8 show that the NPLUSO plasmid has a higher level of activation than the U6/CFREE wildtype plasmid. Figure 8

represents the average normalization results of the two autoradiographs shown in Figures 6 and 7. In general, these results demonstrate a trend that the constructed plasmids (OCT 4, OCT 5, NONOCT 10, NONOCT 13, and NPLUSO 10) have a greater effect on transcriptional activity than the U6/CFREE wildtype plasmid and the dl-84/U6/CFREE plasmid. The normalization results of these transfection experiments were substantially affected by the poor recovery/ expression of the beta-tubulin (BT-68) internal standard. Although this discrepancy did affect the analysis of the experiments, the data was then used to develop transcription experiments to further study the plasmids.

The original transfection results were substantiated by the results of the in vitro transcription assay, which clearly showed that each of the constructed plasmids had a greater effect on transcriptional activity than the U6/CFREE wildtype plasmid and the dl-84/U6/CFREE plasmid. Figure 9 shows the gel separation of the products of the in vitro transcription reactions, indicating that the constructed plasmids have a slightly greater level of activity than the U6/CFREE plasmid (lane 2) and a much greater level of activity than the dl-84/U6/CFREE plasmid (lane 6). Again, the normalization of the signals on this figure provide more accurate and convincing data to support this conclusion. Figure 10 shows a comparison of the normalized signals for all the plasmids used in the transcription assay. In general, it appears that the signals for the experimental plasmids are higher than that of the U6/CFREE plasmid. Since the same elements are found in both the constructed plasmids and the U6/CFREE wildtype plasmid (with the exception of constructed multiple copies), it is likely that this effect is due to the different location of the elements relative to the promoter. Since the U6/CFREE plasmid contains both the OCT and NONOCT elements, it can be compared to the experimental plasmids that contain either one or both of these elements as well. In the wildtype U6/CFREE plasmid, the

beginning of the OCT element is located at position -214 (214 base pairs upstream of the promoter start site). In the constructed plasmids, the insert elements are located much closer to the promoter start site. In the OCT insert plasmids, the 5' boundary of the element is at position -101 relative to the promoter start site. In the NONOCT insert plasmids, the 5' boundary is at position -110, and in the NPLUSO insert plasmids, the 5' element boundary is at position -120. The observation that these experimental plasmids, containing the same type of individual inserts as the U6/CFREE wildtype plasmid, have a higher level of transcription indicates that the location of the elements has a dramatic effect on the activity of the U6 snRNA promoter.

In vivo transfection and in vitro transcription assays indicate that the distal OCT element may have a greater effect on the transcriptional activity of the U6 snRNA promoter than the NONOCT element. Both the in vivo transfection and in vitro transcription experiments showed that the individual elements OCT and NONOCT each activated the level of transcription initiated by the U6 promoter. The second set of in vivo transfection experiments (Figure 7), however, slightly indicated that the OCT element alone (Lanes 5 and 6) had a greater effect on the promoter than the NONOCT element alone (Lanes 9 and 10). Subsequent in vitro transcription reactions substantiated these results by showing greater transcriptional activity for the OCT experimental plasmids than for the NONOCT plasmids (Figure 9). In Figure 9, a comparison of lanes 3, 4, and 5 (OCT plasmids) to lanes 7 and 8 (NONOCT) indicates a higher level of activity for the OCT plasmids. Both sets of OCT and NONOCT plasmids, however, still showed a higher level of transcriptional activity than the dl-84/U6/CFREE control that did not contain any upstream elements.

Experiments demonstrate that the combined OCT and NONOCT element (NPLUSO) has the highest level of transcriptional activation; furthermore, there is an indication that the individual effects of OCT and NONOCT are only additive in this combined element. The initial in vivo transfection experiments indicated a high level of transcriptional activation for the NPLUSO plasmids containing both the OCT and NONOCT elements. These results were repeated for a single NPLUSO plasmid containing the combined OCT-NONOCT element in a reverse orientation in the final set of transfection assays. In Figure 8, the P10 sample representing this plasmid, shows the highest level of activity. In the in vitro transcription assays, two more NPLUSO plasmids were tested. However, one of these plasmids contained an insert that was not correctly linked with the U6 snRNA promoter, and thus no transcription signal was observed (Figure 9, lane 10). The other NPLUSO plasmid (P14) contained multiple copies of the NPLUSO combined element in the reverse orientation. In the initial in vitro transcription assays, both of the useable NPLUSO plasmids showed very high levels transcriptional activity (Figure 9, lanes 9 and 11). The quantitative results in Figure 10 demonstrate that the P10 and P14 samples exhibit a high level of activity in comparison to the other plasmids. In general, this level of activity was higher than that of either the OCT or NONOCT plasmids. Thus, it appears that the signals of the OCT and NONOCT elements simply combine to give a greater signal for the NPLUSO element (Figures 9 and 10).

Based on the different experimental plasmids used, the transcriptional activity of the U6 promoter appears to be independent of upstream element orientation or multiple copies. Based on both transfection and in vitro transcription assays, there appears to be no dramatic effects due to the orientation of the distal elements. While there are some

discrepancies in the original transfection assay results, this result is apparent in the in vitro transcription assay (Figure 9). In the OCT plasmids, the presence of a combined OCT forward and OCT reverse (OCT 4, lane 3) does not appear have a dramatically different effect from the remaining two OCT plasmids that contained reverse OCT elements (lanes 4 and 5). At present, however, the sampling of plasmids for the NONOCT and NPLUSO elements is not sufficient for studying the effects of orientation.

In terms of a dosage effect, the level of transcriptional activity of the U6 promoter also appears independent of the presence of multiple copies of an upstream element. In the OCT plasmids, for example, the plasmid containing four reverse OCT elements (OCT 5, Figure 9, lane 3) does not have a dramatically different signal than the plasmid containing only one OCT reverse (OCT 7, Figure 9, lane 5). For the NONOCT plasmids, the NON 13 plasmid (Figure 9, lane 8) containing two forward NONOCT elements shows a similar signal to the NON 10 plasmid (Figure 9, lane 7), which contains only one forward NONOCT element. Finally, in the NPLUSO plasmids, the P14 plasmid (Figure 9, lane 11) containing four reverse NPLUSO elements does not have a dramatically different signal than the P10 plasmid (Figure 9, lane 9) containing only one reverse NPLUSO element. Thus, these preliminary results demonstrate that the activity of the U6 snRNA promoter is not dramatically influenced by multiple copies of the upstream OCT and NONOCT elements. The presence of single copies of each of these elements will substantially increase the activity level of the U6 promoter; additional copies do not seem to increase transcriptional activity.

DISCUSSION

Previous work has already shown that the distal promoter region of the polymerase III-transcribed U6 snRNA gene used in this study contains two elements, termed OCT and NONOCT, that influence transcription (17). Although fairly unique in Pol III-transcribed genes, this upstream control region is similar to that found in Pol II-transcribed snRNA genes; in both cases, the distal sequence elements are located over 200 base pairs from the start site of transcription (1,2,24,25). This research shows that these two elements in combination result in transcriptional activation of the U6 snRNA promoter when compared with a U6 snRNA promoter lacking these distal elements. In addition, each of these elements individually increases the level of promoter activity, with the OCT element activating the U6 snRNA promoter more than the NONOCT element. However, both of these elements are necessary for the full transcriptional activation of the promoter. The distance of these elements from the promoter is also a definite factor as shown by the differences in promoter activation between the U6/CFREE wildtype plasmid and the constructed experimental plasmids. Finally, element orientation and dosage appear to have no dramatic effects on the level of transcriptional activation.

While the primary purpose of this study was to determine the quantitative transcriptional effects of the OCT and NONOCT elements on the promoter of the polymerase III-transcribed U6 snRNA gene, the ultimate goal is to develop an understanding of the chemical mechanism by which these enhancer-like elements function. Past research has already shown that the OCT element binds to the Oct-1 protein in vitro (9,10). In addition, other researchers found that this Oct-1 protein could facilitate the binding of a transcription factor to the proximal sequence element (PSE) of the snRNA gene (19)(Figure 2). In fact, their

research showed that the purified Oct-1 protein itself could stimulate transcriptional levels in the human 7SK gene, which possesses an octamer motif like the one in the U6 snRNA gene (18, 19). One preliminary mechanism based on this research involves the Oct-1 protein, which binds to the OCT element, promoting the binding of the PSE transcription factor to the proximal promoter region. This would possibly activate an initiation complex and the start of transcription.

In regards to this possible mechanism, this study demonstrates some interesting results in regards to the effects of the distance of the elements from the proximal promoter. Based on the results of the transfection and transcription experiments, it appears as though the normal location of the OCT and NONOCT elements is not required for increased levels of transcription; in fact, the level of activation actually increases when the elements are moved closer to the promoter. One explanation of this phenomenon could be that the OCT and NONOCT elements are moved closer to the proximal promoter naturally by the formation of chromatin in the nucleus. Thus, moving these elements closer to the promoter would naturally cause a greater amount of transcriptional activation. This does not explain, however, why the location of the OCT and NONOCT elements are relatively conserved among different classes of Pol II- and Pol III-transcribed snRNA genes. This conservation of location would tend to support the argument that location does have an effect. It may be necessary for the OCT and NONOCT elements to lie within a conserved distal region that comes within close proximity to the U6 snRNA promoter following the organization of the chromatin within the nucleus. However, the results of this study do not necessarily support this type of theory involving a conserved location. Perhaps the development of these OCT and NONOCT elements is rather recent in evolutionary terms, and the forces of nature have not yet affected their location. If this is the case, then it

might be very possible to change the location of these elements without affecting the activation of the U6 snRNA promoter. Whichever theory is examined, both depend on the hypothesis that the OCT and NONOCT elements must be brought within close proximity of the promoter region to have an effect. The results of this study support this hypothesis, demonstrating that in an experimental plasmid system, the closer these elements are to the proximal promoter region, the greater the transcriptional activation. A more comprehensive understanding of the effects of location could be easily attained through future experimentation. These experiments would involve creating different plasmid constructions containing the OCT and NONOCT elements at incremental distances from the promoter, beginning with the elements being located directly upstream of the proximal promoter region. This would show if a direct relationship between element distance from the promoter and transcriptional activation exists.

In addition to studying the mechanism of the OCT element, recent research has also focused on the role of the NONOCT element. In particular, it has been demonstrated that the NONOCT element sequence is virtually identical to the distal SPH element (SphI Postoctamer Homology) previously found in several chicken snRNA enhancer regions and the upstream region of vertebrate selenocysteine tRNA genes (Kunkel, et al., manuscript submitted). This research demonstrated that the U6/SPH/NONOCT element bound the SPH-binding factor (SBF). In addition, by using mutant SPH/NONOCT elements, it was shown that deviations from the consensus sequence reduced the level of activation of the U6 snRNA promoter. Furthermore, these experiments demonstrated that the mutants did not compete effectively for the SBF protein while it efficiently bound to the wildtype SPH/NONOCT plasmid. Overall, this series of experiments provides preliminary evidence that the SBF protein does act as a transcription factor in that it directly affects the transcriptional levels of the gene. Furthermore, since

SBF binds the SPH/NONOCT element, this provides some insight into a mechanism explaining the effects of the NONOCT element on the U6 snRNA promoter (Kunkel, et al., manuscript submitted).

In terms of this study, the mechanisms of actions of both the OCT and NONOCT elements are particularly interesting with respect to how they affect each other. Already, the potential mechanism for the action of OCT involves the binding of the Oct-1 transcriptional factor, which facilitates the binding of another transcriptional factor to the PSE region of the promoter. Could a similar type of mechanism exist for the NONOCT element, even though it binds the distinct SPH-binding factor? Based on these lines of research, it is interesting to ask if these two mechanisms are linked. The results of this study seem to indicate that the mechanisms of OCT and NONOCT influences are not necessarily linked. Both of these elements activate the U6 snRNA promoter successfully, indicating that they do not depend on each other to function. Furthermore, the results demonstrate that the combined effects of the two elements are additive only, indicating that when present in combination, the OCT and NONOCT elements do not affect the functioning of the other. Altogether, these observations support the conclusion that the mechanisms of the OCT and NONOCT elements seem to function independently. While the OCT element ultimately appears to act upon the PSE (proximal sequence element) through several intermediate transcription factors, it is possible that the NONOCT element acts at a different site within the U6 snRNA promoter.

The results of this study also demonstrate the U6 snRNA promoter activation is not dramatically affected by the orientation or dosage of either the OCT or NONOCT elements. A conclusive analysis of the effects of element orientation is not possible in this study because of the sampling of constructed plasmids. However, the plasmid sampling can be analyzed in terms of element

dosage. In each of the three plasmid groups, OCT, NONOCT, and NPLUSO, there were plasmids containing single and multiple copies of the elements. An analysis of the transfection and in vitro transcription experimental results does not provide any supportive evidence that multiple copies of the elements increase the level of transcriptional activity. In contrast, a previous study of polymerase II-transcribed genes suggested that multiple copies of transcriptional activators may act in a synergistic fashion, meaning that their effects on transcription are greater than additive (26). This effect is not observed in the polymerase III-transcribed U6 gene promoter; in fact, the effect of multiple copies of the same upstream element does not appear to be additive at all. Plasmids containing multiple elements exhibit a similar transcriptional activity as those containing single elements. Could these results indicate a difference in the formation of the polymerase II and polymerase III transcription complexes? Or do they suggest a difference in the mechanism of the snRNA and mRNA promoters? In terms of this question, the study of a polymerase II-transcribed snRNA gene promoter would be interesting.

The results indicating that the effects of OCT and NONOCT are orientation and dosage independent are consistent with the fact that several different types of snRNA and selenocysteine-tRNA promoter distal regions exist that contain these two elements (Figure 11); although most of these regions are fairly similar in position and sequence, their exact construction is quite variable. Nonetheless, they all function to activate the promoter in each of these genes.

Based on background studies and this research, it appears that the mechanism for the functioning of the OCT and NONOCT upstream elements is a rather flexible one. The location of the elements in the upstream region is not restricted such that only one position will activate transcription successfully. Furthermore, orientation and dosage appear to have little effect on the activation

of the promoter. The two distal elements, OCT and NONOCT, do not appear to affect each other's ability to function but do have an additive effect on the level of transcriptional activation of the U6 snRNA promoter. Based on the research presented, these results will help formulate a more accurate model of the mechanism of human U6 small nuclear RNA transcription.

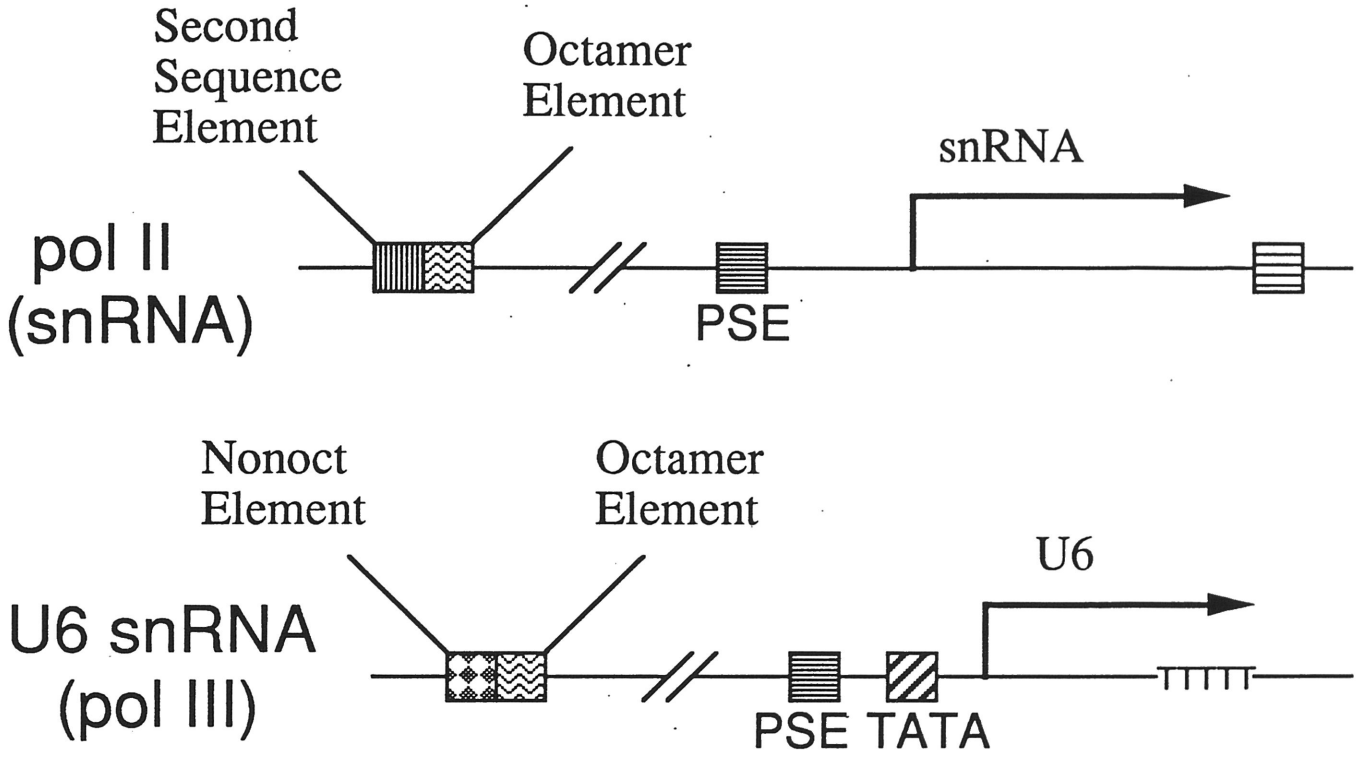


FIGURE 1: Diagram of the distal and proximal gene regions of polymerase II- and polymerase III-transcribed genes. The pol II-transcribed genes contain a PSE element in the proximal region but no TATA element; a conserved octamer element and a second sequence element that varies between pol II-transcribed genes are found in the distal region. The pol III-transcribed human U6 snRNA gene contains PSE and TATA elements in the proximal region and OCT (octamer) and NONOCT elements in the distal region.

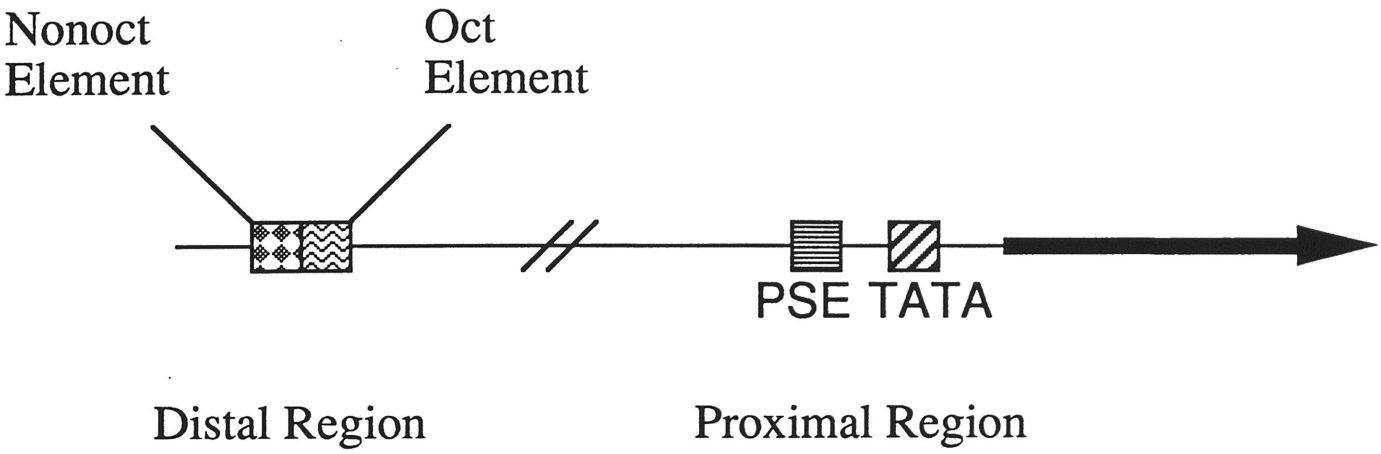
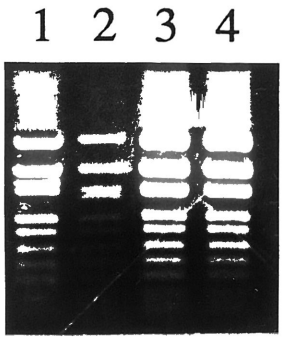


FIGURE 2: Human U6 snRNA gene promoter, including the proximal region, containing the proximal sequence element (PSE) and the T-A rich TATA element, and the distal region, containing the OCT and NONOCT elements.



Lanes:

1: Oct 5, containing multiple Oct inserts
2: Oct 6, containing no insert
3: Oct 7, containing a single Oct insert
4: Oct 8, containing a single Oct insert

FIGURE 3: Restriction map of several OCT plasmids with MspI to determine the presence of the OCT element in the plasmid insert. Using the negative plasmid in lane 2 as a control, the fourth band of the restricted plasmids in lanes 1, 3, and 4 is migrating slower, indicating that this insert is larger and contains the OCT oligonucleotide. In lane 1, this band is significantly larger than normal, indicating the presence of multiple copies of OCT in the insert.

Table 1

OCT Oligonucleotide:

GATCCATATTTGCATAT
GTATAAACGTATACTAG

NONOCT Oligonucleotide:

GATCAGGGCCTATTTCCCATGATTCCTTCA
TCCCGGATAAAGGGTACTAAGGAAGTCTAG

NPLUSO Oligonucleotide:

GATCCTATTTCCCATGATTCCTTCATATTTGCATAT
GATAAAGGGTACTAAGGAAGTATAAACGTATACTAG

Constructed Plasmids:

OCT Plasmids:

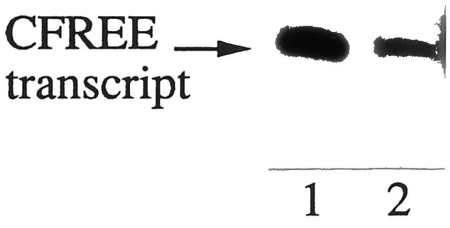
OCT 4:	One forward OCT oligonucleotide and one reverse OCT oligonucleotide
OCT 5:	Four reverse OCT oligonucleotides
OCT 7:	One reverse OCT oligonucleotide

NONOCT Plasmids:

NON 10:	One forward NONOCT oligonucleotide
NON 13:	Two forward NONOCT oligonucleotides

NPLUSO Plasmids:

P10:	One reverse NPLUSO oligonucleotide
P13:	One forward NPLUSO oligonucleotide, but not successfully attached to the dl-84 promoter element
P14:	Four reverse NPLUSO oligonucleotides

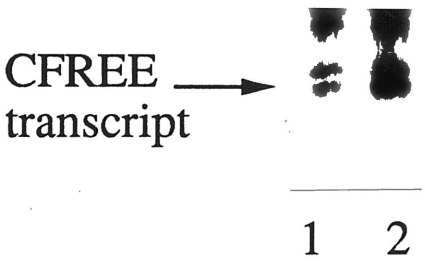


Lanes:

1: U6/CFREE plasmid

2: dl-84/U6/CFREE plasmid

FIGURE 4: In vivo transfection experiments show that the U6/CFREE plasmid containing the distal region with the OCT and NONOCT elements has a higher level of transcriptional activity than the dl-84/U6/CFREE plasmid. This assay shows a higher CFREE transcription signal on a polyacrylamide gel for the U6/CFREE plasmid (lane 1) than for the dl-84/U6/CFREE plasmid (lane 2).

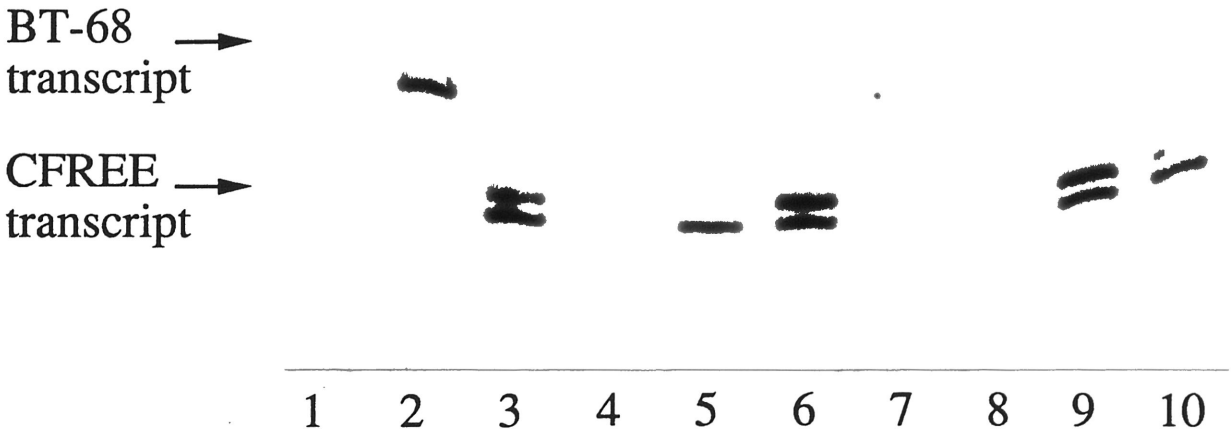


Lanes:

1: dl-84/U6/CFREE plasmid

2: U6/CFREE plasmid

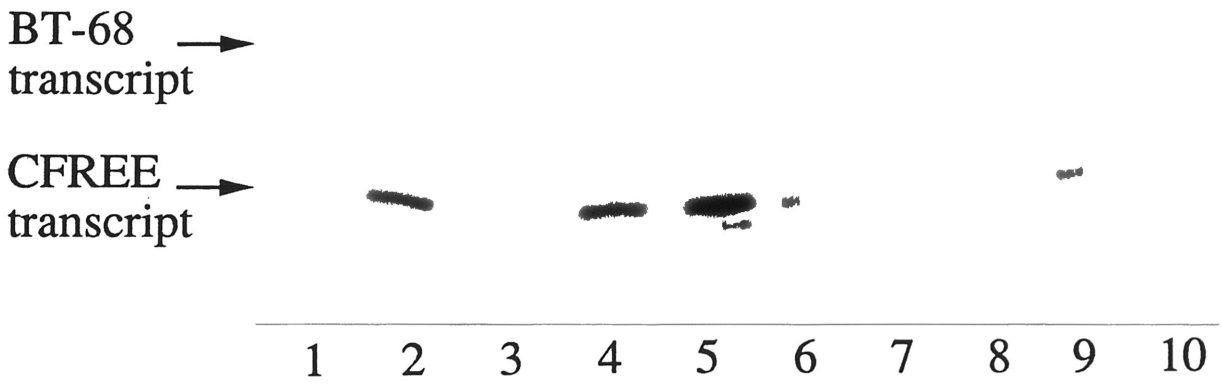
FIGURE 5: In vitro transcription experiments show that the U6/CFREE plasmid containing the distal region with the OCT and NONOCT elements has a higher level of transcriptional activity that the dl-84/U6/CFREE plasmid. This assay shows a higher CFREE transcription signal on a polyacrylamide gel for the U6/CFREE plasmid (lane 2) than for the dl-84/U6/CFREE plasmid (lane 1). The results of this experiment support those seen in Figure 4 for an in vivo transfection experiment.



Lanes:

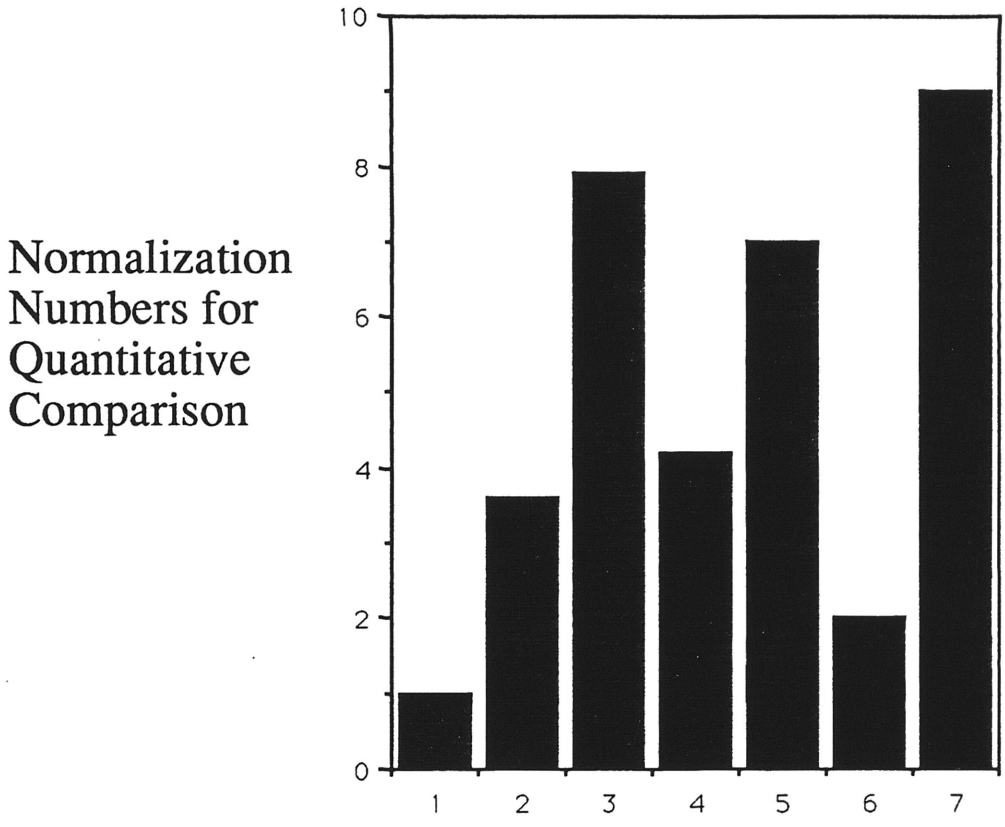
1: P10 plasmid containing one NPLUSO insert, reverse
2: pGEM plasmid marker, restricted with MspI
3: U6/CFREE plasmid containing wildtype distal region
4: X
5: OCT 4 plasmid containing two OCT inserts, forward and reverse
6: OCT 5 plasmid containing four OCT inserts, reverse
7: OCT 7 plasmid containing one OCT insert, reverse
8: dl-84/U6/CFREE plasmid containing no distal region
9: NON 10 plasmid containing one NONOCT insert, forward
10: NON 13 plasmid containing two NONOCT inserts, forward

FIGURE 6: The results of this in vivo transfection assay visibly indicate that the constructed OCT (lanes 5, 6) and NONOCT (lanes 9, 10) plasmids increase the level of transcription over that of the dl-84/U6/CFREE plasmid (lane 8). Furthermore, the quantitative normalization shown in Figure 8 uses the BT-68 transcript as an internal standard to show that these OCT and NONOCT plasmids have a higher level of transcription than the U6/CFREE plasmid as well. The results of lanes 1, 4, and 7 were not analyzed because of poor recovery/expression leading to inaccurate normalization.



Lanes:
1: pGEM plasmid marker, restricted with MspI
2: U6/CFREE plasmid containing wildtype distal region
3: X
4: P10 plasmid containing one NPLUSO insert, reverse
5: OCT 4 plasmid containing two OCT inserts, forward and reverse
6: OCT 5 plasmid containing four OCT inserts, reverse
7: OCT 7 plasmid containing one OCT insert, reverse
8: dl-84/U6/CFREE plasmid containing no distal region
9: NON 10 plasmid containing one NONOCT insert, forward
10: NON 13 plasmid containing two NONOCT inserts, forward

FIGURE 7: The results of this transfection assay are similar to those shown in Figure 6. The OCT (lanes 6, 7) and NONOCT (lanes 9, 10) plasmid visibly show a higher level of transcription than the dl-84/U6/CFREE plasmid (lane 8). In addition, however, the NPLUSO plasmid (lane 4) shows a high level of transcription. Quantitative normalization in Figure 8 demonstrates that, in general, the constructed OCT, NONOCT, and NPLUSO plasmids have higher transcription levels than both the dl-84/U6/CFREE and U6/CFREE plasmids. The results of lanes 3 and 7 were not analyzed due to poor recovery/expression.



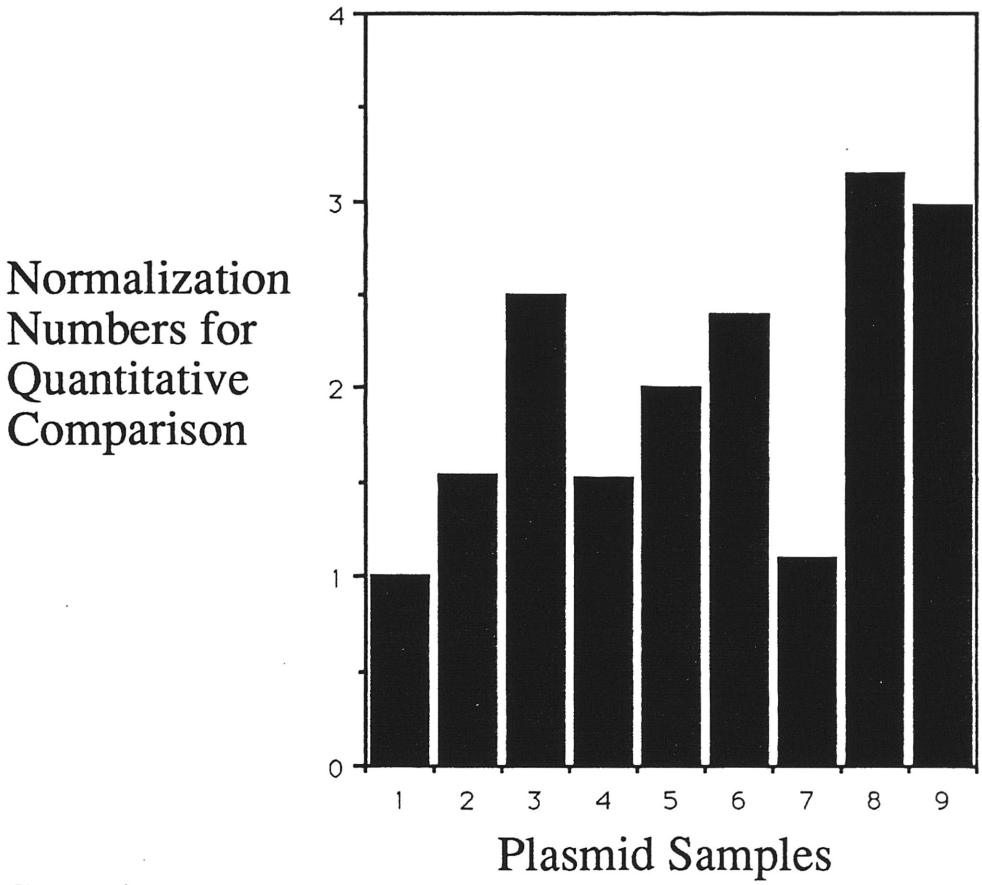
Samples:

1. dl-84/U6/CFREE plasmid containing no distal region
2. U6/CFREE plasmid containing wildtype distal region
3. OCT 4 plasmid containing two OCT inserts, forward and reverse
4. OCT 5 plasmid containing four OCT inserts, reverse
5. NONOCT 10 plasmid containing one NONOCT insert, forward
6. NONOCT 13 plasmid containing two NONOCT inserts, forward
7. P10 plasmid containing one NPLUSO insert, reverse

FIGURE 8: This graph represents the average quantitation numbers of two transfection experiments. The U6/CFREE (2) plasmid again has higher activity that the dl-84/U6/CFREE (1) plasmid. Furthermore, the OCT and NONOCT plasmids (3-6) have higher activity than both the U6/CFREE and dl-84/U6/CFREE in general. The NPLUSO plasmid, however, had the highest level of activity (7).

Lanes:
1: dl-84/U6/CFREE plasmid containing no distal region
2: U6/CFREE plasmid containing wildtype distal region
3: OCT 4 plasmid containing two OCT inserts, forward and reverse
4: OCT 5 plasmid containing four OCT inserts, reverse
5: OCT 7 plasmid containing one OCT insert, reverse
6: dl-84/U6/CFREE plasmid containing no distal region
7: NON 10 plasmid containing one NONOCT insert, forward
8: NON 13 plasmid containing two NONOCT inserts, forward
9: P10 plasmid containing one NPLUSO insert, reverse
10: P13 plasmid containing one NPLUSO insert, forward, but not attached to dl-84 promoter element
11: P14 plasmid containing four NPLUSO inserts, reverse

FIGURE 9: In vitro transcription assays show that the OCT, NONOCT, and NPLUSO plasmids (lanes 3-5, 7-11) have greater activity than dl-84/U6/CFREE and U6/CFREE. Furthermore, the OCT plasmids (3, 4, 5) appear to have greater activity than the NONOCT plasmids (7, 8), and the NPLUSO plasmids (9, 11) are more active than both of these, indicating an additive effect.



Samples:

1. dl-84/U6/CFREE plasmid containing no distal region
2. U6/CFREE plasmid containing wildtype distal region
3. OCT 4 plasmid containing two OCT inserts, forward and reverse
4. OCT 5 plasmid containing four OCT inserts, reverse
5. OCT 7 plasmid containing one OCT insert, reverse
6. NONOCT 10 plasmid containing one NONOCT insert, forward
7. NONOCT 13 plasmid containing two NONOCT inserts, forward
8. P10 plasmid containing one NPLUSO insert, reverse
9. P14 plasmid containing four NPLUSO inserts, reverse

FIGURE 10: This graph represent the normalized quantitation numbers for an in vitro transcription assay. Along with Figure 9, this graph demonstrates that the OCT plasmids (3, 4, 5) have slightly greater activity than the NONOCT plasmids (6, 7). Furthermore, the NPLUSO plasmids (8, 9) containing both the OCT and NONOCT elements have the greatest level of activity, indicating an additive effect.

Human U6 snRNA:

-239 TTC CCA tgA TtC CTt cat (0 nts) OCT reverse

Chicken U1 snRNA:

-186 cTC CCg GCA TGC agC GCG (7 nts) OCT reverse

Chicken U4B snRNA:

OCT reverse (5 nts) -205 TTC CCA GCA TGC CTC GCG

Bovine tRNA (Ser)Sec:

-228 TTC CCA GaA TGC gcg GCG (2 nts) OCT forward

X. laevis tRNA (Ser)Sec:

-212 gTa CCA GCA TGC CTC GCG (no OCT element)

FIGURE 11: Different types of snRNA and selenocysteine-tRNA gene promoters all possess the OCT and NONOCT (SPH) elements but in a variety of orientations and relative locations. The comparative table above shows the SPH (or NONOCT) sequence for several snRNA and selenocysteine-tRNA gene promoters, along with the relative location of the OCT element. The consensus SPH sequence is: TTC CCA GCA TGC CTC GCG.

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