Identification of a Tripartite Basal Promoter Which Regulates Human Terminal Deoxynucleotidyl Transferase Gene Expression*

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Dipa Bhaumik†, Baoli Yang, Theoni Trangas§, Jeffrey S. Bartlett, Mary Sue Coleman, and David H. Sorscher¶

From the Department of Biochemistry and Biobiophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7260

In order to locate the promoter region of the human terminal deoxynucleotidyl transferase gene, serially truncated segments of the 5'-flanking region of the gene were cloned into a chloramphenicol acetyltransferase reporter vector. Transient transfection analyses of the terminal transfection-reporter gene constructs identified the basal promoter region within -34 to +40 base pairs relative to the transcription start site. Three promoter elements were defined in this region. The primary element is within 34 base pairs upstream of the transcription start site. The CAP site is 62 base pairs upstream of the translation start site. The secondary element involves sequences around the transcription start site. The third is located 25 base pairs downstream from the initiation site (+25 to +40). This tripartite basal promoter was not tissue specific; similar patterns of promoter activity were observed in terminal transfection expressing and non-expressing cells. Transfection analyses also indicated the presence of negative regulatory elements upstream of the basal promoter region, and these elements were preferentially active in cells expressing terminal transfectase.

Immunoglobulin (Ig) and T-cell receptor gene assembly is attained through gene rearrangement of a large number of separately coded variable (V), diversity (D), and joining (J) gene segments (Ikuta et al., 1992). It is well known that the diversity of the immune repertoire is greatly increased by the addition of nucleotides (N regions) at the D/J and V/D junctions during Ig and T-cell receptor gene rearrangements (Desiderio et al., 1984; Schatz and Baltimore, 1988). Terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase, that randomly polymerizes deoxyribonucleoside triphosphates onto initiator DNAs (Bollum, 1974) has been shown to be the only enzyme involved in N region addition (Smale and Baltimore, 1989). A number of studies have revealed that several of the TATA-less genes contain a DNA sequence (termed the initiator element or Inr) that encompasses the transcription start site, and is responsible for specifying the precise initiation site for transcription (Zenzie-Gregory et al., 1992; Weis and Reinberg, 1992). A number of studies have revealed that several of the TATA-less genes contain a DNA sequence (termed the initiator element or Inr) that encompasses the transcription start site, and is responsible for specifying the precise initiation site for transcription (Zenzie-Gregory et al., 1992; Weis and Reinberg, 1992). In TATA-less promoters the Inr element plays an important role in the correct positioning of RNA polymerase II. The human TdT gene, which lacks a TATA box and binding sites for common transcription factors, like Sp1, contains an Inr element that is responsible for the basal transcription of the gene. (Smale and Baltimore, 1989). The human TdT gene also lacks a consensus TATA box, but visual inspection of the 5' proximal sequences of the gene failed to locate an obvious Inr element. The 5' upstream sequences of human and mouse TdT do share some homology. Alignment of the 5' sequences of the human and mouse TdT genes revealed 72% homology within a region corresponding to -186 to +51 bp in the mouse gene when three insertions of 9, 20, and 27 bp of the human sequence were excluded (Lo et al., 1991). Interestingly, our study shows that the 27-bp insertion corresponds precisely to the position of the transcription start site of the human gene identified in vivo.

This limited homology between the 5' sequences of the murine and the human TdT genes coupled with the apparent absence of a mouse Inr element homolog suggested that the mouse and the human TdT genes employed different promoter elements. Our goal was to study the 5' sequences in detail and to identify control elements participating in human TdT gene regulation. We have demonstrated that transcription of the human TdT gene initiated at a single nucleotide. Transient transfection analyses of the human TdT gene promoter region identified three requisite elements located upstream of the start site, at the start site and interestingly, downstream of the start site that were necessary for correct initiation and optimal promoter activity.

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† Present address: Dept. of Pathology, Stanford University School of Medicine, Stanford, CA 94305-5324.
‡ Present address: Papanikolaou Research Center, Hellenic Anti-Cancer Institute, 171 Alexandras Ave., Athens 11594, Greece.
§ To whom correspondence should be addressed. Tel.: 919-866-6292; Fax: 919-866-2952.
¶ The abbreviations used are: TdT, terminal deoxynucleotidyl transferase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; bp, base pairs; Inr, initiator; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pairs.

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A fragment containing exon-1 of TdT was subcloned into the SmaI site of the human TdT. The 5' flanking regions of human TdT were cloned by blunt-end ligation into the HindIII-PstI site of pCAT-Enhancer (Promega) to produce the -286CAT, -202CAT, -54CAT, -34CAT, -5CAT, +25CAT, and +41CAT vectors were constructed by first subcloning a 995-bp BamHI fragment of the coding region of the human TdT into the HindIII-PstI site of pUC13. A clone with the fragment in the reverse orientation was then digested by HindIIIPstI and EcoRI or BamHIIAccI site of PUC13. The reverse primer used was R1 which encompasses the translational initiation (721 or 421 bp upstream of the ATG) was cloned into the BamHI site of pUC13 constructed by amplifying the appropriate region by using the po- lymerase chain reaction (PCR) and sequencing. The RACE (RACE1, RACE2, RACE3, RACE4, and RACE5) PCR products were then subcloned into the HindIII-PstI sites of pCAT-Enhancer (Promega) to produce the -930CAT DNA. The numbering for the vectors corresponds to the distance in base pairs upstream or downstream from the TDt transcription start site. The vectors containing -665CAT and -305CAT were constructed similarly, except that a BamHI/EcoRI or a BamHI/AccI fragment (721 or 421 bp upstream of the ATG) was cloned into the BamHI/EcoRI or BamHI/AccI site of pUC13. The -286CAT, -202CAT, -54CAT, -34CAT, -5CAT, +25CAT, +41CAT, and -54 to +25CAT vectors were constructed by amplifying the appropriate region by using the polymerase chain reaction (PCR) and then subcloning the DNA fragments containing oligonucleotide primers (Fig. 2) which contained a HindIII site in the 5' primers and PstI site in the 3' primers were used in the reactions. The amplified products were then subcloned into the HindIII/PstI sites of pCAT-Enhancer. The constructs -286CAT, -202CAT, and -54CAT, the reverse primer used was R1 which encompasses the translational start site. The reverse primer R2 was used for making the constructs -34CAT, -5CAT, +25CAT, and +41CAT. These constructs included 55 bp of exon-1. R1 was used as the reverse primer to make -54 to +25 CAT. The -286CAT(R) DNA (−286 bp upstream of the ATG) was cloned into the BamHI/EcoRI or BamHI/AccI site of pUC13. The -286CAT vector contained an insertion of 200 µdCTP. Five µl of the DNA containing the added DNA was amplified by PCR using 20 pmol of both the appropriate internal antisense primer and BRL anchor primer. After RNase digestion the cDNA was isolated and incubated with TdT media supplemented with 10% fetal bovine serum (Trangas and Colonello, 1989). Translation (U.S. Biochemical Corp., Cleveland, OH) was performed in the presence of 100 µCi of [35S]methionine and [35S]cysteine. The cell-free extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. The sizes of the products were determined by comparison with 32P-end-labeled φX174/HaeIII markers and DNA sequencing ladders.

RESULTS

Mapping of the Transcription Start Site of the Human TdT Gene—Standard methods for determining the transcription start site of the human TdT gene were unsuccessful in our hands since this gene is transcribed at a very low rate in vivo. Therefore, we sought to identify the transcription start site for the human TdT gene by using the powerful 5'-RACE technique. This highly sensitive technique employs isolation of RNA, synthesis of cDNA, addition of C residues to the cDNA, amplification of the product by PCR, and sequencing of this product. To ensure that the template in these reactions was RNA, a series of primers, each of which produced a different length of amplified product was used (Fig. 1A). PCR products of the predicted size were observed when RNA from the TdT-positive cell line NALM-6 was used in the reaction (Fig. 1B, lanes 1, 2, and 5–7). The sizes of the PCR products detected in each lane were consistent with a transcription start site of approximately 50–75 bases 5' to the ATG translational start site. In contrast, RNA from the TdT-negative cell line KOPN-8 yielded no specific PCR

MATERIALS AND METHODS

Cell Lines—The cells used in this study were established cell lines of leukemic lymphoma origin and were found to represent different, early stages of lymphocyte maturation (Davidson et al., 1985). The human lymphoid cell lines KT-1, MOLT4, (pre-T), NALM-6 (pre-B) which expressed TdT and KOPN-8 (pre-B), KE-37 (mature-T cells) that did not express TdT were used. The cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (Trangas and Colonello, 1989).

Isolation of RNA and 5'-RACE—RNA was isolated from cells by gua- nidine isothiocyanate cell lysis and centrifugation through CsCl cushion (Sambrook et al., 1989). 5'-RACE reactions were as described by Bethesda Research Laboratories: cDNA was synthesized using 2 µmol of antisense primer (RACE5 or RACE4, see below) and 1 µg of total RNA. After cDNA synthesis, the cDNA was isolated and incubated with TdT and 200 µdCTP. Five µl of the DNA containing the added DNA sequences was amplified by PCR using 20 pmol of both the appropriate internal antisense primer and BRL anchor primer in a volume of 50 µl. PCR products were separated on an agarose gel and were sized by comparison with the migration of HindIII-digested φX174 DNA markers (Saunders et al., 1988) except the BRL anchor primer.

Construction of TDTCAT Plasmids—The -930CAT DNA was con- structed by first subcloning a 995-bp BamHI fragment of the 5' non-coding region of the human TdT (995 bp upstream of the ATG) into the BamHI site of pUC13. pUC13 is a pUC-based plasmid in which the multiple cloning site has been modified in our laboratory to facilitate the subcloning of 5'-flanking regions of TdT. A HindIII/PstI fragment from pUC13 containing the 995 bp of TdT was then subcloned into the HindIII/PstI site of pCAT-Enhancer (Promega) to produce the -930CAT DNA. The numbering for the vectors corresponds to the distance in base pairs upstream or downstream from the TDt transcription start site. The vectors containing -665CAT and -305CAT were constructed similarly, except that a BamHI/EcoRI or a BamHI/AccI fragment (721 or 421 bp upstream of the ATG) was cloned into the BamHI/EcoRI or BamHI/AccI site of pUC13. The -286CAT, -202CAT, -54CAT, -34CAT, -5CAT, +25CAT, +41CAT, and -54 to +25CAT vectors were constructed by amplifying the appropriate region by using the polymerase chain reaction (PCR) and sequencing. The -930CAT DNA was amplified by PCR using 20 pmol of both the appropriate internal antisense primer (RACE5, RACE4, see below) and 1 µg of total RNA.

Primer Extension Analysis—Forty-eight h after transfection cells (5 x 105 to 106) were harvested, and total RNA was isolated by the guanidinium-CsCl method (Sambrook et al., 1989). For primer extension 50 µg of total RNA was annealed to 32P-end-labeled oligonucleotide -5'GGGATATATCAATGGTATATC-3'. This oligonucleotide is complementary to the CAT coding region beginning 43 bp downstream of the translational start site. To anneal primer and target RNA, samples were heated for 8 min at 85 °C in 30 µl of annealing buffer (0.25 mM KCl, 10 mM Tris-HCl, pH 8.0, 2.5 µl of 10 mg/ml butyryl-CoA (Sigma) and 0.1 µCi of [35S]chloramphenicol (52 mCi/mmol, Dupont-New England Nuclear), and reactions were incubated at 37 °C. Depending on the cell line used the reaction time was varied from 15 min to 1 h to keep product formation in the linear range. The reactions were terminated by adding 300 µl of xylene, and the amplified products were isolated by the phase extraction procedure of Seed and Shenoy (1988). Primer extension was expressed as counts/minute per hour of radioactive butyryl chloramphenicol. These numbers were then normalized for equal transcription efficiency within a given cell type by calculating the CAT activity/100 units of β-galactosidase activity. The normalized data for a particular construct, for transfection experiments conducted on different days, varied by 2-fold. Data for the CAT assays were expressed as means ± SD of four to seven independent transfection experiments using at least two different plasmid preparations of the same construct.

PCR Amplification—PCR products of the predicted size were resuspended in reverse transcription buffer (50 mM Tris-HCl, pH 8.0, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol) and 0.5 mM deoxynucleotides. After addition of 20 µCi of [32P]dATP and 100 units Moloney murine leukemia virus reverse transcriptase (BRL), samples were incubated for 60 min at 37 °C. cDNA products were incubated with 20 µg/ml RNase (Ambion) for 30 min and then treated with proteinase K before phenol-chloroform extraction and ethanol precipitation. Samples were analyzed on a 6% polyacrylamide, 8 M urea denaturing sequencing gel. Primer extension products were visualized by autoradiography, and the sizes of the products were determined by comparison with 32P-end-labeled φX174/HaeIII markers and DNA sequencing ladders.
products in this experiment (Fig. 1B, lanes 3, 4, 8, and 9). The precise transcription start site (a T) at 62 bp 5' upstream of ATG was determined by cloning and sequencing one of the PCR products.

To confirm the size of the 5' non-translated region of the RNA, another experiment was performed. 32P-End-labeled oligonucleotides complementary to sequences upstream or downstream of the putative transcription start site were hybridized to mRNA from TdT-positive cells by using Northern analysis. These experiments showed that oligonucleotides antisense to mRNA sequences downstream but not to DNA sequences upstream of the proposed transcription start site hybridized to the RNA and were detected on the Northern blots (data not shown).

Functional Analysis of the 5'-Flanking Sequence of the Human TdT Gene—In this study we sought to establish a functional assay for potential promoter elements in the 5' upstream region of the human TdT gene. This region was sequenced when the gene was cloned (Riley et al., 1988). Within 995 bp upstream from the translation start site ATG, a number of known transcription factor-binding sites were present (Fig. 2). AP2-binding sites were identified in the regions −55 to −62 and +33 to +41. There was an Ets-binding site at −101 to −95 which in the murine TdT gene has been shown to be essential for transcription (Ernst et al., 1993). There were several binding sites for INF-1, a binding site for Oct-1, and a TATA box. In order to test the function of these elements in vivo, a transient transfection assay was developed.

The cells we elected to use for these experiments were undifferentiated lymphoblastoid cells that were well established in continuous suspension cell culture. The TdT-positive lines used were MOLT-4, NALM-6, and KT-1 cells. The TdT-negative lymphoblastoid line was KE-37. These permanent cell lines are the only source for TdT expression (Drexler et al., 1985) and are highly resistant to transfection (Lo et al., 1991).

Calcium phosphate, DEAE-dextran, and lipofection transfection protocols failed to promote efficient transfer of control plasmids. By contrast, efficient transfection of all cell lines was obtained by electroporation following optimization of the density of the cells at the time of harvest, the cell concentration, buffer composition of the cell suspension to be electroporated, and the temperature at which the cell suspension was kept prior to electroporation.

Using the optimized electroporation protocol, a series of CAT constructs containing various lengths of the 5'-flanking sequences of the human TdT gene was transiently transfected into the KT-1, MOLT-4, or NALM-6 cells (TdT expressing) or the KE-37 (TdT non-expressing) cells. The CAT activity was measured 48-h post-transfection. CAT activities were corrected for transfection efficiencies by cotransfecting the cells with pCMVβ DNA and normalizing the CAT activity/100 units of β-galactosidase activity. The results from the first series of constructs are shown in Fig. 3. Identical data were observed with all three positive cell lines, but for simplicity, only the results obtained with KT-1 cells are illustrated in the figure.
from the transcription start site and surrounding the start site were analyzed by primer extension. The RNAs employed in primer extension analysis were isolated from transfected cells. The results of two representative experiments are shown in Fig. 5. KE-37 and KT-1 cells were transfected with the constructs −54CAT (Fig. 5, lanes 2, 5, 7, and 9), +25CAT (Fig. 5, lanes 3 and 6), and −54 to +25CAT (Fig. 5, lanes 8 and 10). The control DNA in these experiments was the basic vector pCAT-Enhancer (Fig. 5, lanes 1 and 4). The RNA employed in the primer extension reaction was isolated from cells after 48 h in culture. The predicted cDNA reaction product from −54CAT was 171 bp long. The vector sequence contributed 109 bp, and the transcription start was expected to be at about 62 bp from the translation start site. A major band was visible corresponding to 70 bp from the ATG when primer extension analysis was done on RNA isolated from −54CAT-transfected cells. This site mapped closely (8 bases longer) to the transcription start site determined by 5′-RACE (Fig. 1) and by in vitro transcription assay (data not shown). The difference in the transcription start site is probably due to technical details of the assays. Manipulation of the TdT mRNA during the 5′-RACE procedure could easily result in loss of a few bases. Alternatively, the sizes of the cDNA products measured after primer extension could be slightly inaccurate due to lane to lane variability in the electrophoresis procedure. The doublet observed at the major start site might represent transcription initiation at either of two bases. Alternatively, the doublet is an artifact of the primer extension reaction. A faint band (at least 4-fold less intense than the major band) was also observed at 42 bp upstream from ATG when −54CAT was used as the transfecting DNA. Both of these major and minor bands were consistently absent in analyses of RNA isolated from cells transfected with the +25CAT construct. This result suggested that sequences upstream from the start site and surrounding the start site were important for correct initiation of transcription.

The construct containing sequences spanning −54 to +25 was found to initiate transcription accurately since a cDNA product of the expected size (131 bp) was observed after transfection of this DNA construct into KT-1 and KE-37 cells (Fig. 5, lanes 8 and 10). These data indicated that accurate transcription could occur only when two parts of the basal promoter were present. The primer extension analysis of CAT mRNA from transfected cells coupled with the results of the CAT assay (Fig. 4) sug-
suggested that all three segments of the promoter (the primary element within \(-34\) bp, the element spanning the transcription start site and the downstream element) were necessary for optimal promoter activity.

DISCUSSION

An important step in identification of promoter elements is an accurate positioning of transcription start. Previously, Koiwai and Morita (1988) had identified two transcription start sites for the human TdT gene, at 80 and at 106 bp upstream from the ATG. However, our in vivo experiments (5'-RACE technique) consistently identified a single start site at 62 bp upstream from translation start. While the reasons for the discrepancy are not clear the transfection experiments support the site we identified.

The human TdT gene lacks TATA and CAAT consensus sequences immediately 5' to the initiation site, a characteristic that is shared by other eukaryotic genes. The 5'-flanking se-
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FIG. 5. Primer extension analysis of RNA derived from TdT-CAT DNA-transfected cells. Primer extension reactions were performed on 50 µg of total RNA isolated from KE-37 and KT-1 cells transfected with 35 µg of the indicated plasmid DNA. 5'-end-labeled oligonucleotide complementary to the CAT coding region ("Materials and Methods") was used for primer extension. The cDNA reaction products were electrophoresed on a denaturing gel, and the products were visualized by autoradiography. In lanes 1–6, the upper arrow indicates the predicted +1 transcription start site, and the lower arrow indicates a minor transcription start site. The control lane contains the primer extension data with 50 µg of RNA isolated from cells transfected with pCAT-Enhancer. Lanes 7–10 were from another experiment. The upper arrow and lower arrow in lanes 7–10 both indicate the predicted +1 transcription start site. The cDNA products in lanes 8 and 10 were 40 bp shorter than those in lanes 7 and 9 because of the deletion of sequences from +25 to ATG during cloning of the −54 to +25CAT construct. HaeIII-digested 0X174 size markers (32P-labeled) are shown on the left side of each panel. The dashes, −, on the left of the figure correspond to the bands in the marker lane and represent 281, 271, 234, 194, and 118 bases, respectively.

sequences of the human and mouse TdT genes share limited homology. The mouse TdT gene was difficult to study because transcription rates of this gene were very low (Smale and Baltimore, 1989; Lo et al., 1991). No CAT activity was detected when TdT/CAT fusion plasmids were used for transfections of mouse lymphoma cells (Lo et al., 1991). Therefore, to identify regulatory elements, Smale and co-workers resorted to the use of replicating vectors in transient transfection assays (Lo et al., 1991; Ernst et al., 1993). However, the authors of that study suggested replicating vectors probably masked all but the strongest control elements. We initially confronted a similar problem in our study of the human TdT gene. Transient transfection assays using CAT reporter vectors fused to different regions of the human TdT gene were difficult to establish. However, optimizing conditions for electroporation helped to increase the transfection efficiency and made these experiments feasible.

With transient transfection assays of immature lymphoid cells firmly established, we tested a series of TdT/CAT fusion genes in transfection assays. The optimal levels of CAT activity were consistently observed when the sequence spanning −54/−34 to ATG was used in transfection assays. Further analyses identified the basal promoter of the human TdT gene within the region −34 to +40 bp. Moreover, three distinct control elements in this region were identified. The first control element was located 34 bp upstream from the transcription start site, but we have not yet determined its precise boundaries. Negative regulation was also evident upstream from −54 bp. This transcription inhibition effect was far more prominent in TdT-positive than in TdT-negative cell lines. In fact, when the −930CAT and −656CAT DNA were used to transfect KT-1 cells, virtually no CAT activity was detected. The same constructs yielded 25 times more CAT activity in TdT-negative than in TdT-positive cells. With the construct −356CAT, three times more CAT activity was detected in TdT-negative than in TdT-positive cells. These results suggest that a negative regulatory element exists in the region −356 to −656 and that it is differentially active in the TdT-positive cells. Inspection of the 5' sequence for putative transcription factor-binding sites identified potential AP1, INF-1, NF-1, and Oct-1 elements in this region (−356 to −656). Any one of these or a different protein binding within this region could possibly exert a negative effect on TdT transcription in TdT-positive cells. The sequences between −54 to −356 also exerted negative regulation, and the effect was roughly equivalent in both KT-1 and KE-37 cells. A variety of negative elements, in different gene promoters has been characterized (Banerjee et al., 1987; Wang and Brand, 1990; Rincon-Limas et al., 1991). The exact position and nature of the negative elements in human TdT 5' sequences will be the subject of further investigation.

The second of the three elements within the human basal promoter was found to span the transcription start site. When a construct was used that contained only 5 bp upstream from the transcription start site along with downstream sequences to the ATG, CAT activity was readily detected although at a reduced level. Furthermore, no specific transcripts were observed in primer extension experiments when sequences both upstream and around the start site were removed. The fact that this construct (−5CAT) was able to drive CAT activity, and that transcription in vivo initiated at a single nucleotide in the
absence of a TATA sequence indicated that an Inr type element was present in the human promoter sequence (Weis and Einstein, 1992; Kollmar and Farnham, 1993). There was no visible homology between the human TdT initiation site and the mouse TdT Inr element, but we did observe a similarity to an 8-bp Inr consensus sequence (GCAGTCTC) described by Kollmar and Farnham (1993). In order to match the Inr homology to our human TdT sequence, we had to place the start site at an A (as described in Kollmar and Farnham, 1993) two nucleotides upstream from the T initiation site detected by 5'-RACE.

The third promoter element spanned nucleotides +25 to +40. This element alone was not capable of initiating transcription correctly. Primer extension analyses of the transcription products indicated that initiation was no longer at a single nucleotide when only the third promoter element was contained in the constructs. However, removal of this sequence in the CAT transcription (Zenzie-Gregory et al., 1993). In order to match the Inr homology to our human TdT sequence, we had to place the start site at an A (as described in Kollmar and Farnham, 1993) two nucleotides upstream from the T initiation site detected by 5'-RACE.

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In summary, we have demonstrated that the human TdT gene contains a basal promoter that functions comparably in both TdT expressing and non-expressing cells. The basal promoter of the human TdT gene is not identical to that found in the murine TdT gene. The latter seems to consist only of an Inr element. In addition, an LyP-1 and an Ets sequence upstream from the Inr seems to provide lymphoid specificity to the mouse TdT gene. In contrast, the human TdT basal promoter consists of three elements, including a consensus Inr element. While LyP-1- and Ets-binding sites are present upstream from the human basal promoter (Lo et al., 1991; Ernst et al., 1993), participation of these two proteins in lymphoid-specific transcription of the human TdT gene is not apparent from the experiments we have performed since these sequences had no detectable effect on CAT activity.

Studies of the boundaries of the basal promoter, negative regulatory elements, and tissue-specific enhancer elements in other regions of the human gene are currently underway in our laboratory. These results will help us understand transcriptional regulation of the human TdT gene and will help define the various mechanisms by which tissue- and differentiation-specific gene expression is controlled.

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