MOLECULAR BIOLOGY OF TERMINAL TRANSFERASE

Authors: Lucy M. S. Chang
F. J. Bollum
Department of Biochemistry
Uniformed Services University of the
Health Sciences
Bethesda, Maryland

Referee: Robert C. Gallo
Laboratory of Tumor Cell Biology
National Cancer Institute
Bethesda, Maryland

I. INTRODUCTION

Terminal deoxynucleotidyl transferase activity was first described in 1960[1] as a side reaction present in partially purified DNA polymerase preparations from calf thymus glands. The activity was shown to polymerize deoxynucleotides onto a performed initiator without template direction.[2] At that time, the terminal addition activity was presumed to be related to DNA polymerase as some form of active subunit. Terminal transferase was subsequently demonstrated to be a unique entity by tissue localization,[3,4] developmental[3] and immunological[5] studies. It is clear today that terminal transferase is indeed a most unusual DNA polymerase found only in prelymphocytes in early stages of lymphoid differentiation. Since immunoglobulin[6,7] and T-cell receptor gene rearrangements[8,9] occur in prelymphocytes during the time when these cells express terminal transferase, this enzyme has been postulated[2,3,10-12] to participate in generating immunological diversification.

The restriction of normal tissue localization of the enzyme to specific thymus[3] and bone marrow[4] cells, and the detection of the expanded terminal transferase population occurring in acute leukemias resulted in practical rewards from basic research that were totally unpredicted. Today, immunochemical[13,14] and enzymatic determinations of terminal transferase[15] provide the basis for diagnostic classification of human lymphoid leukemia in the pre-B and pre-T lineages[16,17] and differential diagnosis of myeloid leukemias. The cell biology of terminal transferase and uses of terminal transferase as a marker in leukemia diagnosis are subjects of earlier reviews[16,17]

Employment of terminal transferase to modify DNA molecules by adding homopolymer tails[18,19] provided the first test tube recombinant DNAs. The practical rewards of this feat are now manifest. This review concentrates on the uses of terminal transferase as a reagent in molecular biology, the biochemical properties, and the molecular biology of terminal transferase.

II. SYNTHETIC PROPERTIES OF TERMINAL TRANSFERASE

A. Requirements of Terminal Transferase Reactions

Terminal transferase requires a deoxynucleoside triphosphate (dNTP) as an activated monomer, an oligodeoxynucleotide initiator with minimum chain length of three and a free 3′-hydroxyl group, and a divalent cation.[2] When the dNTP to be polymer-

ized is a purine, best rates are obtained with Mg^{+2}. When the dNTP used is a pyrimidine, best rates are obtained with Co^{+2}. Oligodeoxynucleotides of any base composition can be used providing the 3′-hydroxyl is present and the chain length is greater than or equal to three. A typical terminal transferase reaction contains 0.2 *M* potassium cacodylate buffer at pH 7.2, 1 m*M* 2-mercaptoethanol, 4 m*M* $MgCl_2$, 1 m*M* dATP or dGTP, 0.01 m*M* $d(pN)_6$ and 750 units (one unit is equal to one nmole of dATP polymerized per hr) per m*l* of terminal transferase. When the monomer is 1 m*M* dCTP or dTTP, 1 m*M* $CoCl_2$ is used instead of $MgCl_2$ and the 2-mercaptoethanol is added last to avoid metal precipitation. Bovine serum albumin is generally added to increase total protein concentration to at least 50 μg/m*l* to reduce adsorption to test tube surfaces.

B. Synthesis of Homopolydeoxynucleotides

The optimum conditions to be used for the synthesis of polydeoxynucleotides by terminal transferase will depend on composition of the polymer as well as the chain length desired.[20] Since terminal transferase polymerizes mononucleotides in a distributive manner and does not carry out the reverse reaction, the chain length of the polymer at the completion of the reaction should be equal to the input monomer:initiator (M/I) ratio, with a Poisson distribution in mol wt. The reaction conditions described above have an M/I ratio of 100 and will result in an average chain length of 100 in the product. To produce chain lengths from 100 to 1000 initiator concentrations are reduced and dNTP concentrations are increased to set the desired M/I ratio. The reaction should reach completion in time predicted from the amount of enzyme activity present.

Synthesis of polydeoxynucleotides with even greater average chain lengths can generally be achieved by simply increasing the monomer:initiator ratios; although, some care must be taken to ensure that the reaction will approach completion. Assuming sufficient enzyme activity, time, and pure substrates, a number of other factors can influence the final yield (and thereby, chain length). The products of the terminal transferase reaction are polydeoxynucleotide and inorganic pyrophosphate. Inorganic pyrophosphate forms insoluble compounds with both Mg^{+2} and Co^{+2} and removes cation from solution. To synthesize extremely long homopolymers the monomer concentration can be increased to at least 10 m*M* and the divalent cation concentration is increased stoichiometrically. When the synthesis is carried out with a greater than 4 m*M* dNTP reaction conditions should be modified to allow the divalent cation to remain in solution through the course of the synthesis. EDTA and many other metal complexers inhibit enzyme activity[21] and cannot be used. In dATP and dGTP polymerization reactions, yeast inorganic pyrophosphatase is included so that inorganic pyrophosphate generated will be continuously converted to orthophosphate. When dTTP or dCTP and $CoCl_2$ is used, 0.1 *M* Glycine:NaOH at pH 7.0 should be included in the reaction mixture to provide a safe means of maintaining the solubility of Co^{+2} during the polymerization reaction.

For example, to synthesize poly (dA) with an average chain length of 10,000, a reaction is setup containing 0.2 *M* potassium cacodylate at pH 7.2, 10 m*M* dATP, 0.001 m*M* $d(pA)_6$, 10 m*M* $MgCl_2$, 1 m*M* 2-mercaptoethanol, 750 units/m*l* of terminal transferase, 50 μg/m*l* bovine serum albumin and 1 unit/m*l* of yeast inorganic phosphatase (Worthington), and incubated at 35°C for 24 hr. The progress of the reaction can be monitored by hypochromicity or production of inorganic phosphate. Analysis of the poly (dA) product synthesized in this manner shows the expected narrow chain length distribution, averaging around 10,000 nucleotides.

Synthesis of poly (dA) and poly (dT) with chain lengths up to 20,000 nucleotides with terminal transferase have not been a problem. Synthesis of poly (dC) with chain lengths up to 3,000 nucleotides also proceeds reasonably well, but larger polymers of

deoxycytidylate are more difficult (even when cobalt pyrophosphate precipitation is inhibited with glycine as described above) because of the tendency to form double stranded associations at neutral pH[2]. Synthesis of poly (dG) chains greater than 25 nucleotide long is impossible since oligo (dG) aggregates,[22] resulting in the 3′-hydroxyl group of the growing polymer becoming relatively less accessible to further chain growth. The aggregation properties of dG polymers provided the basis for producing nuclease resistant products in assaying terminal transferase activity in crude tissue extracts.[3] To produce high polymers of dG, dNacGTP is used as the monomer in the reaction to produce poly (dNacdG).[22] Poly (dG) can be obtained by deacylation of poly (dNacdG) with concentrated NH_4OH.

C. Synthesis of Oligodeoxynucleotides

Oligodeoxynucleotides of defined chain length can be prepared by enzymatic degradation of polydeoxynucleotides followed by separation of DEAE-cellulose columns.[23] Oligonucleotides with more than one base can also be synthesized directly by terminal transferase[24] using judicous selection of M/I ratios and applying the principles of polymer chemistry. The basic principle used for mol wt prediction in oligonucleotide and polymer synthesis is the same, assuming that the addition of nucleotides onto the growing chain is distributive. The average distribution of product chain lengths at equilibrium must be the input monomer:initiator ratio if terminal transferase exhibits no preference for initiators of different chain lengths and base composition. But even in the simpliest two substrate reaction catalyzed by terminal transferase one of the substrates (the growing chain) is changed with each round of reaction by addition of one additional nucleotide. Small differences in the affinity of the enzyme for the changing substrate population can produce major differences in the final distribution of products. This is particularily true for short oligodeoxynucleotide initiators. The Km of terminal transferase for oligodeoxyadenylates ($d(pA)_n$) when n=3 is three times greater than when n=4, the Km for n=4 is 2 times greater than when n=6, and the Km for n=6 is about the same as when n=7 or 8.[25] When $d(pA)_4$ is used as an initiator for dATP polymerization the chain length distribution of the product is found to be much broader than when $d(pA)_6$ is used. In addition to the initiator chain length bias of the enzyme, it is also necessary to consider the preference of the enzyme for the dNTP being polymerized. For example, the product distribution in the polymerization of dATP onto $d(pA)_4$ (input monomer:initiator ratio of three) was found to be biased toward longer chain lengths. The statistical distribution expected can be obtained using a dATP analogue such as dNacATP. Similarly, polymerization of dTTP onto $d(pT)_4$ produced products biased toward longer chain lengths, while polymerization of dCTP onto $d(pT)_4$ produced a distribution approaching the Poisson. By examining the Kms of terminal transferase for the substrates (both initiator and monomer) to be used, it is possible to predict the shape of the product distribution.

The fact that terminal transferase can polymerize dNTPs onto the 3′-hydroxyl group of short oligodeoxynucleotides raises the possibility that the enzyme could be used for step-wise synthesis of DNA with defined sequence. If terminal transferase could polymerize dNTPs with blocked 3′-hydroxyl groups, sequential addition of specific deoxynucleotides would be relatively simple. 3′-OAcdNTPs have been tested as substrates for terminal transferase and no reaction was observed.[26] Since terminal transferase can utilize rNTP,[27,28] ddNTP,[29] and Cordycepin triphosphate[30] for terminal addition, as well as dUTP,[31] methyl-,[32] *N*-acetyl-,[22] *N*-anisoyl-,[33] and *N*-benzoyl-derivatives[33] of dNTPs as substrates, a systematic study of modified dNTPs that might allow single step addition by terminal transferase still remains a possibility for providing an enzymatic method for synthesis of nucleic acids of specific sequences. With cloned terminal

transferase[34] and expression in *E. coli*[35] site specific mutagenesis might now allow modification of the enzyme in a way that would allow single step additions.

D. Modification of Natural DNA with Terminal Transferase

Since the first demonstration of the use of terminal transferase in cloning P22 DNA[18] and SV40 DNA[19] fragments, the terminal addition reaction is now used extensively for joining DNA molecules. Homopolymer tailing of DNA fragments with 3′-protruding ends with terminal transferase does not present any unusual problems, although the specificity of the enzyme for the base composition at the 3′-end of DNA and the monomer being added must be taken into consideration in order to obtain the predicted distribution of products. DNA molecules with blunt or recessive ends cannot be tailed by terminal transferase with the predicted product distribution since terminal transferase cannot interact distributively with the 3′-hydroxyl present in a double stranded structure. If the 3′-hydroxyl groups of DNA molecules with blunt or recessive ends are exposed by treatment of the DNA molecules by λ-exonuclease[18,19] efficient homopolymer tailing by terminal transferase is obtained.

Many recipes have been devised for tailing DNA molecules with blunt or 3′-recessive ends, including the use of Co^{+2},[36] Mg^{+2} in low ionic strength buffers,[37] or the use of Mn^{+2} [38] in the terminal transferase reaction. These conditions have been described in other reviews[39,40] on this subject. Michelson and Orkin[41] showed that even in the presence of Co^{+2}, tailing of blunt-ended DNA substrate is inefficient and the products are biased toward longer additions.

The use of analogue substrates with terminal transferase does provide a means to label the 3′-end of polynucleotides. Chain termination occurs when one residue of ddNMP[29] or Cordycepin monophosphate,[30] or a maximum of three rNMP[28] are added to the 3′-hydroxyl group of a polydeoxynucleotide. Although the affinity of the enzyme for these analogue substrates and their reaction rates are low, complete end labeling can be obtained. 3′-End labeling with terminal transferase also provides a means of determining oligodeoxynucleotide and polydeoxynucleotide chain lengths.

Since terminal transferase catalyzes polymerization of dNTP without template direction, modification of the 3′-terminus of a polydeoxynucleotide with a mismatched nucleotide at a specific site of a recombinant DNA molecule should result in site specific mutagenesis.[42] Site specific mutagenesis can be achieved by controlled incorporation of mismatched monomers (i.e., setting the terminal transferase reaction conditions so that only one or few mismatched nucleotides are incorporated) followed by in vitro repair of the "mutagenized" DNA with DNA polymerases lacking associated 3′-exonuclease. AMV reverse transcriptase[43] and mammalian DNA polymerase-β[44] have been shown to be able to utilize initiator-template systems containing mismatched 3′-terminal nucleotides. Although conditions are available to allow efficient tailing of noncomplementary nucleotides onto DNA, efficient incorporation of one or two nucleotides by terminal transferase requires a *free* 3′-hydroxyl. In most cases this can be accomplished by denaturing the DNA prior to the terminal transferase reaction and setting up the terminal transferase reaction with a dNTP:DNA ratio of 1 to 2, followed by reannealing of the terminal transferase product for repair synthesis.

An alternative method for generation of site specific mutations would be the use of α-S-dNTPs as monomers for tailing DNA molecules. The conditions and procedures used for this are described in another review.[42]

III. STRUCTURE OF TERMINAL TRANSFERASE PROTEIN

The terminal transferase referred to up to this point and commonly used for synthetic purposes is the enzyme isolated by conventional purification from calf thymus

glands. Calf thymus gland provides an easily available source for large-scale preparation of this enzyme. Although enzyme preparations free of degradative activities were available in 1964,[45] purification of terminal transferase to homogeneity was finally achieved in 1971.[46] The homogeneous terminal transferase isolated by conventional purification has a native mol wt of 32,000 and consists of 2 peptides, an 8 kDa α-peptide and a 26 kDa β-peptide. These preparations are found to be active in polymerization of dNTPs onto deoxynucleotide initiators with a minimum chain length of three, and contain no associated enzyme activities. Calf thymus terminal transferase was subsequently purified in other laboratories with a similar two peptide structure having somewhat different mol wt.[47] The development of rabbit antibody against the homogeneous enzyme[5] and immunoblot procedures[48] allowed examination of the native peptide structure of terminal transferase present in crude extracts of mammalian tissue. It is now apparent that the original homogeneous terminal transferase represents a highly degraded enzyme, and different degrees of proteolysis might account for slight differences in the mol wt of the final purified material obtained in different laboratories.

The earliest indication that the purified terminal transferase might be a degradation product came from the observation that newly synthesized terminal transferase in human lymphoblastoid cells contained only a single peptide with mol wt of 58,000.[49] Human terminal transferase purified from lymphoblasts of leukemic patients to near homogeneity by Deibel and Coleman,[47] was also found to be a protein with a single polypeptide of 62,000 kDa. Since antibodies to calf terminal transferase cross-react with the enzyme from other vertebrate species,[5] we reexamined the terminal transferase peptide structure in crude extracts of several tissues and species containing terminal transferase by gel electrophoresis in the presence of sodium dodecyl sulfate followed by immunoblotting. The major molecular species present in all crude extracts has a mol wt of about 58,000.[50] This finding was the final proof that the original homogeneous terminal transferase must contain degraded peptides. Analysis of the purification procedure for calf thymus terminal transferase confirms the fact that proteolysis occurs throughout purification of the enzyme with site specific cleavages producing 56 kDa, 44 kDa, and 42 kDa peptides still carrying the active sites from the 58 kDa parent molecule.[51] Further degradation of the 42 kDa peptide results in formation of the α- and β-peptides present in the low mol wt form of the enzyme. Both of these peptides are required for enzyme activity. The endogenous proteolysis of terminal transferase can be mimicked by the action of trypsin and this data is shown in the autoradiograph of a terminal transferase activity gel in Figure 1. Proteolysis of terminal transferase is not restricted to the calf thymus system since active terminal transferase preparations from human sources are also found to contain degraded peptides.[52,53]

It is not entirely clear at present whether proteolysis of terminal transferase is caused solely by artifactual exposure during purification or whether it is part of the natural process regulating enzyme activity in vivo. Our new immunoaffinity purification procedures allow the rapid isolation of undegraded terminal transferase peptides from bovine and human sources, as well as the low mol wt form from calf thymus glands.[54]

A. Immunoaffinity Purification of Low Molecular Weight Terminal Transferase from Calf Thymus Glands

Quantitative conversion of high mol wt terminal transferase peptides in calf thymus gland extracts to low mol wt peptides occur during batch absorption and elution of the enzyme from phosphocellulose. The enzyme in the active phosphocellulose fractions is concentrated by ammonium sulfate precipitation to produce the equivalent of the AS-II fraction.[45] Endogenous protease activities appear to concentrate on the phosphocellulose and effect the conversion to 32 kDa form. No enzyme activity is lost during this

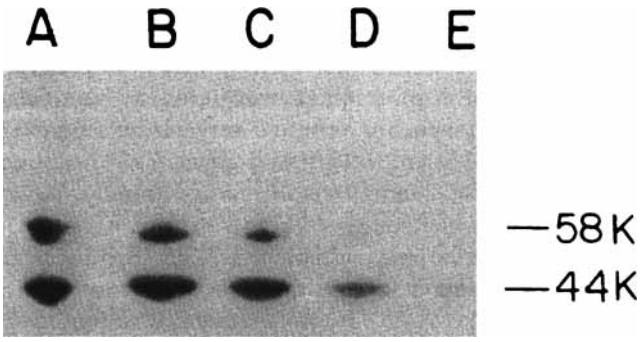

FIGURE 1. Renaturation of trypsin-generated terminal transferase peptides. High molecular weight calf thymus terminal transferase at 200 μg/m*l* was incubated with 20 μg/m*l* of trypsin at 35°C in 50 m*M* Hepes buffer at pH 7.5. Samples were removed at 0 min (lane A), 30 sec (lane B), 1 min (lane C), 2 min (lane D), and 30 min, and trypsin activity was terminated with soybean trypsin inhibitor. The terminal transferase peptides were separated by polyacrylamide gel electrophoresis and the enzyme was renatured and assayed directly on the gel. After the terminal transferase reaction, the gel was washed to remove unused substrates and reaction products were detected by autoradiography. No net loss of enzyme activity was detected during trypsin degradation prior to denaturation for gel electrophoresis. The only terminal transferase peptides that can be renatured following polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate migrate as 58, 56, 44, and 42 kDa peptides. Tryptic peptides having molecular weights less than 42 kDa do not exhibit renaturation in the gels.

preliminary purification step, and in fact some increase in total activity may occur. The redissolved ammonium sulfate precipitate fraction (about 40 mg protein per milliliter) is diluted with 10 volumes of phosphate buffered saline and loaded directly onto a 40 m*l* mouse monoclonal antibody; Protein A Sepharose column (containing 60 mg of mouse IgG_1 against calf thymus terminal transferase covalently linked to Protein A Sepharose) at a flow rate of 4 m*l*/min. The antibody column is washed extensively with phosphate buffered saline, then with 200 m*l* of 0.2 *M* NaCl. Protein is eluted from the column with 1 *M* $MgCl_2$ at about 1 m*l*/min. Fractions containing enzyme activity are pooled and dialyzed overnight against 3 × 1.2 *l* of 0.1 *M* NaCl in 50 m*M* Tris:HCl at pH 8.0 and once against 500 m*l* of 500 m*M* potassium phosphate at pH 7.4 in 1 m*M* 2-mercaptoethanol containing 50% glycerol. The dialyzed enzyme is stored at −20°C with no loss in enzyme activity after storage for at least 2 years. A summary of this purification scheme is presented in Table 1A.

The low mol wt form of terminal transferase purified by this new procedure is extremely stable on storage and has reaction properties identical to those described for conventionally purified enzyme. No conventional endo- or exonuclease activity can be detected. This form of enzyme is most useful as an enzyme reagent in recombinant DNA research. Analysis of peptides present in the immunoaffinity purified fraction by gel electrophoresis in the presence of sodium dodecyl sulfate shows that greater than 95% of the protein has the α- and β-peptide structure and the remainder is present as the 42 kDa species (Figure 7, lane c).

B. Purification of High Molecular Weight Terminal Transferase from Calf Thymus Glands

All operations are carried out at 4°. Frozen calf thymus glands (950 g) are homoge-

Table 1
PURIFICATION OF TERMINAL TRANSFERASE FROM CALF THYMUS GLANDS AND HUMAN LYMPHOBLASTOID CELLS

	Fraction	Volume (ml)	Protein (mg)	Sp.Ac. (units/mg)	T. E. A. units
A.	Low molecular weight terminal transferase from calf thymus glands				
	AS-II	76	4,710	656	3.1×10^6
	$MgCl_2$ Eluate (dialyzed)	60	36	98,400	3.6×10^6
B.	High molecular weight terminal transferase from calf thymus glands				
	Crude extract	3810	49,500	37*	1.8×10^6†
	Protamine sulfate supernatant	3750	31,900	54†	1.7×10^6†
	$MgCl_2$ Eluate (dialyzed)	52	43	5240	0.22×10^6
	Protein A Sepharose pass-through	57	6.2	34,900	0.22×10^6
	$(NH_4)_2SO_4$ Concentrate	2.1	5.3	37,500	0.20×10^6
	Dialysis vs 3 *M* urea buffer	2.3	4.6	36,600	0.17×10^6
	Chromatofocusing column pool I	4.8	0.91	24,500	22,300
	pool II	9.8	0.83	56,900	47,400
C.	Terminal transferase from human lymphoblastoid cells (KM-3 cell line)				
	Crude extract	180	864	95†	82,300†
	$MgCl_2$ Eluate (dialyzed)	14	0.53	64,700†	34,400†

* Enzyme assays were carried out using (8-^{3}H) dGTP as substrate; other enzyme activities listed in this table were obtained using (8-^{3}H) dATP as substrate. One dATP unit is equivalent to 4 dGTP units.

nized in a Waring blender in 4 *l* of 0.04 *M* potassium phosphate at pH 7.4, 0.04 *M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride, and 1% dimethylsulfoxide. A cloudy supernatant solution was obtained by centrifugation of the homogenate for 15 min at 4,500 rpm in an H4000 rotor in a Sorvall®3B centrifuge. The supernatant solution is filtered through four layers of cheesecloth to remove fat particles and 190 m*l* of 3% protamine sulfate solution is added gradually with good mixing. Stirring is continued for 15 min and the precipitate is allowed to aggregate for 15 min. The precipitate is removed by centrifugation at 8,500 rpm for 30 min in a GS-3 rotor in a Sorvall®5B centrifuge.

The protamine sulfate supernatant is filtered again using a fluted filter paper and loaded directly onto a 20 m*l* mouse monoclonal antibody:Protein A Sepharose column (containing 30 mg of mouse IgG_1 against calf thymus terminal transferase) at a flow rate of about 8 m*l*/min. The column is washed extensively with homogenization buffer followed by 100 m*l* of 0.2 *M* NaCl. Protein is eluted from the column with 1 *M* $MgCl_2$. Active fractions are pooled, dialyzed overnight against 3×1.2 *l* of 0.1 *M* NaCl containing 50 m*M* Tris:HCl at pH 8.0, and then passed directly through a 20 m*l* Protein A Sepharose column (previously equilibrated with dialysis buffer) at 2 m*l*/min to remove bovine IgG that also bound to the first column. The Protein A Sepharose pass-through is dialyzed against 70% saturated ammonium sulfate made up in 50 m*M* potassium phosphate at pH 7.4 containing 1 m*M* 2-mercaptoethanol. The ammonium sulfate precipitate is collected by centrifugation, redissolved in 50 m*M* potassium phosphate at pH 7.4 containing 1 m*M* 2-mercaptoethanol and stored at −20°C.

The immunoaffinity purified calf thymus terminal transferase prepared directly from crude extracts usually contains a mixture of terminal transferase peptides (Figure 2, lane A). To obtain pure 58 kDa calf thymus terminal transferase, the antibody column purified calf thymus enzyme fraction is subjected to chromatofocusing on a Pharmacia PBE-94 exchanger. The affinity purified calf thymus enzyme fraction is

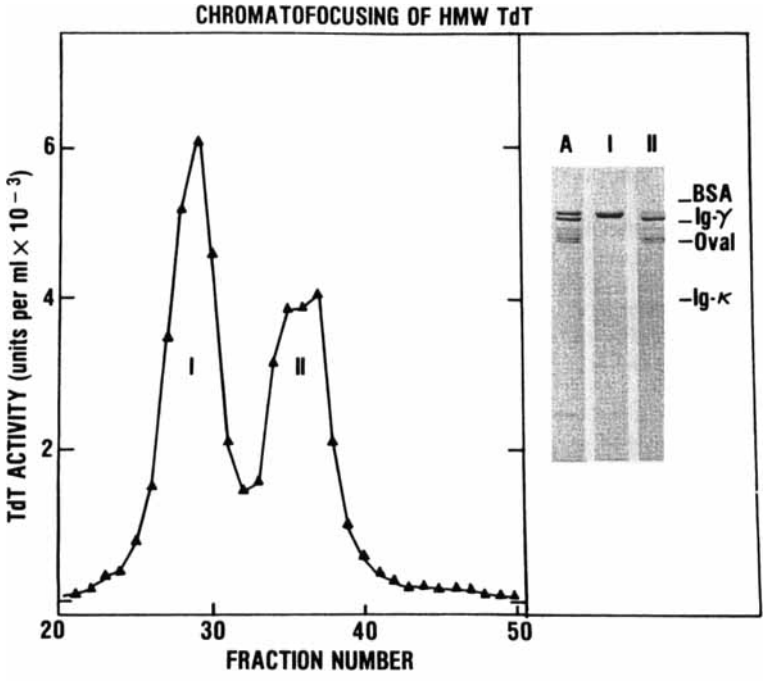

FIGURE 2. Chromatofocusing profile of the high molecular weight species of calf thymus terminal transferase purified by immunoaffinity chromatography. The panel on the right shows the Coomassie Blue stained sodium dodecyl sulfate gel of immunoaffinity purified high molecular weight calf thymus terminal transferase (lane A), chromatofocusing column pool I (lane I), and chromatofocusing column pool II (lane II).

thawed and dialyzed for 6 hr against 500 mℓ of 10 mM Tris:HCl at pH 8.0, 1 mM dithiothreitol, 3 M urea and 20% glycerol. Any turbidity formed is removed by centrifugation. A 12 mℓ (1 × 12 cm) PBE-94 column with a 1 cm pad of Sephadex ® G-50 layered on the top is prepared and equilibrated with 15 column volumes of 0.025 M ethanolamine:acetate buffer at pH 9.4 (starting buffer). Just before loading the sample, 2.5 mℓ of elution buffer (1 in 10 dilution of pH 6.0 Polybuffer 96-CH_3COOH in 3 M urea with 1 mM dithiothreitol, 20% glycerol) is passed onto the column. The sample is then loaded and eluted with 120 mℓ of elution buffer at a flow rate of about 10 mℓ/hr.

The pH gradient obtained on the chromatofocusing column ranges from 8.8 to 6.4. The first peak of terminal transferase activity elutes at pH 8.1 and the second peak at pH 7.6. After the fractions are assayed active fractions are pooled and dialyzed overnight against 100 volumes of 50 mM potassium phosphate at pH 7.4 containing 1 mM 2-mercaptoethanol and 20% glycerol and then against a solution of 70% saturated ammonium sulfate. The protein precipitated with ammonium sulfate is collected by centrifugation and redissolved in 50 mM potassium phosphate at pH 7.4 containing 1 mM 2-mercaptoethanol and stored at −20°. A summary of the purification procedure for the high mol wt calf thymus terminal transferase is presented in Table 1B.

Gel analysis of the immunoaffinity purified high mol wt calf thymus terminal transferase fraction shows the presence of the 58 kDa and 56 kDa doublet, the 44 kDa, and 42 kDa doublet as well as a small amount of nonterminal transferase peptides around 46 to 48 kDa (Figure 2, lane A). The 58 kDa peptide is obtained in pure form by subjecting the immunoaffinity purified enzyme fraction to chromatofocusing as de-

scribed above. The separation obtained on this column is shown in Figure 2. The 58 kDa peptide (Figure 2, lane I) elutes at more alkaline pH (peak I) than the 56 kDa, 44 kDa, and 42 kDa peptides (Figure 2, lane II) that elute in peak II (Figure 2). Urea (3 *M)* was used in the chromatofocusing buffer to maintain solubility of the 58 kDa peptide. The high mol wt terminal transferase fractions obtained from the chromatofocusing column appear to have lower than expected specific activity (Table 1B) on a molecular basis. We do not know if this is a property of the 58 kDa species or whether inactivation has occurred during the separation process. Although the yield of active enzyme is rather low, this procedure does provide a method for obtaining pure 58 kDa calf thymus terminal transferase that can be used for more detailed characterization of reaction properties, amino acid composition, and primary sequence analysis.

Preliminary characterization of the 58 kDa terminal transferase from calf thymus[54] indicated that the major reaction properties are quite similar to the low mol wt form. No gross differences were observed in the Kms for dNTPs between the native and the low mol wt form of calf thymus terminal transferase. The Km of the 58 kDa form for $d(pA)_{50}$ was found to be two- to fourfold lower than the 32 kDa form of the enzyme depending on the ionic strength used in the reactions. The affinity of the two forms of enzymes for Pst 1 cleaved pBR322 was about the same while the affinity of the 58 kDa form of Eco R1 cleaved pBR322 DNA was four- to sixfold greater than that of the 32 kDa form. Whether the native 58 kDa terminal transferase has different properties for modification of DNA-like molecules will require more detailed characterization of the reaction properties of this enzyme.

C. Immunoaffinity Purification of Terminal Transferase from Human Lymphoblastoid Cells

All operations are carried out at 4°. Eighteen grams of frozen cell pellets of human lymphoblastoid cells (KM-3) were homogenized in 180 m*l* of 0.25 *M* potassium phosphate at pH 7.4 with a Polytron Homogenizer. The homogenate was clarified by centrifugation at 15,000 rpm in a SS-34 rotor in a Sorvall®5B centrifuge, followed by centrifugation for 1 hr at 40,000 rpm in a Ti-60 rotor in a Beckman ultracentrifuge. The supernatant was filtered through a layer of fluted filter paper and loaded directly onto a 1.5 m*l* immunoabsorbent column containing about 3 mg of mouse monoclonal antibody to human terminal transferase covalently linked to Protein A Sepharose. The flow rate was about 1 m*l*/min. After loading, the column is washed with 100 m*l* 0.25 *M* potassium phosphate at pH 7.4 followed by 50 m*l* of 0.5 *M* NaCl in 50 m*M* Tris:HCl at pH 8.0. Protein bound to the column is eluted with 3.2 *M* $MgCl_2$ in 50 m*M* Tris-HCl at pH 8.0 at a flow rate of 0.33 m*l*/min. Active fractions are pooled and dialyzed against three changes of 1.25 *l* of 0.1 *M* NaCl in 50 m*M* Tris:HCl at pH 8.0, once against 50 m*M* potassium phosphate at pH 7.4, 1 m*M* 2-mercaptoethanol in 50% glycerol, and stored at −20°. A summary of the purification of the human terminal transferase from KM-3 cells is presented in Table 1C.

Immunoaffinity purification of terminal transferase from the human cell extract is essentially a one step process since the extracts do not contain immunoglobulins that bind to the column. Terminal transferase in human lymphoblastoid cells is mostly nuclear and appears to be more tightly bound to the chromatin than the enzyme present in the calf thymus gland. The isotonic buffer used in the calf thymus extraction is not adequate for solubilization of the enzyme from human tissue culture cells. The enzyme can be extracted with 0.25 *M* potassium phosphate at pH 7.4, and other proteins that bind nonspecifically to the mouse antibody column may also be extracted. In order to remove these nonspecifically bound proteins we selected a mouse monoclonal antibody to human enzyme that permits a high ionic strength buffer wash of the resin prior to

elution of the enzyme. A higher concentration of $MgCl_2$ is required to elute the human enzyme from this monoclonal column. Although the unbuffered 1 *M* $MgCl_2$ used for the purification of the calf thymus enzyme does not appear to affect the recovery of that enzyme activity from the immunoadsorbents, buffered 3.2 *M* $MgCl_2$ was required for significant recovery of the 58 kDa enzyme from the monoclonal antibody column used for the human cell extracts. Greater than 90% of the terminal transferase activity in the crude extract of KM-3 cells was bound to the resin of which about 50% was recovered in the dialyzed $MgCl_2$ eluate. Sodium dodecyl sulfate polyacrylamide gel analysis demonstrated the predominant peptide present in the immunoaffinity purified human transferase to be the 58 kDa species (Figure 7, lane b). The minor species present may represent the phosphorylated form of terminal transferase.[55,56]

D. Amino Acid Sequence of Human and Bovine Terminal Transferase

Crossreactivity of rabbit anticalf thymus terminal transferase with terminal transferase from other species[5] suggested that terminal transferase protein might be highly conserved through evolution. Polyclonal antibody to the bovine enzyme reacts equally well with human, rodent, and avian terminal transferases. In addition to the conservation of antigenic determinants, immunoblotting studies demonstrated that the mol wt of this enzyme is also conserved in different species.[50] This conservation of protein structure has permitted isolation of terminal transferase cDNA sequences from human and murine cells using rabbit antibodies to calf thymus terminal transferase and cloning vectors that produce fused proteins of *E. coli* β-galactosidase. The availability of large amounts of bovine enzyme from immunoaffinity purification facilitated amino acid sequencing and the homology in amino acid sequences provided conclusive identification of the cDNA for human terminal transferase.[34] The complete cDNA coding sequence has now been determined for human terminal transferase,[35] and the translation of the cDNA sequence provides the complete amino acid sequence for the human enzyme. Figure 3 shows the translated amino acid sequence for human terminal transferase and a comparison with the amino acid sequences determined for a number of peptides sequenced from the bovine enzyme. The extensive homology of the human and bovine enzyme allows us to suggest several features of this protein that may apply generally.

Comparison of the translated human terminal transferase sequence with amino acid sequences determined for calf thymus terminal transferase peptides (Figure 3) shows greater than 90% homology. Of the 263 amino acid residues sequenced from the calf enzyme, only 24 mismatches with the translated human amino acid sequence were found. Edman degradation of the 58 kDa bovine peptide failed on several attempts indicating that the N-terminal residue might be blocked. Sequencing of the 56 kDa bovine peptide produced a sequence of 24 amino acids beginning 22 amino acids in from the N-terminal methionine and having four mismatches with the translated human sequence.

Examination of the complete amino acid sequence now available for the human terminal transferase shows some interesting features that may provide explanation of several puzzling observations concerning the behavior of this enzyme in cells and in solution. Cytological studies on terminal transferase indicate a nuclear localization in bone marrow lymphocytes and leukemic lymphoblasts,[13,16] and a nuclear *and* cytoplasmic localization in cortical thymocytes.[13] Enzyme purified from human lymphoblastoid cells is mostly 58 kDa while a mixture of terminal transferase peptides is always found in thymus preparations. It is possible that the native peptide might have signals for nuclear localization that are removed by proteolysis. Residues 11 to 17 at the N-terminus of the translated human sequence are -Pro-Arg-Lys-Lys-Arg-Pro-Arg-, representing a potential nuclear localization sequence for this protein.[57] Conversion of 58

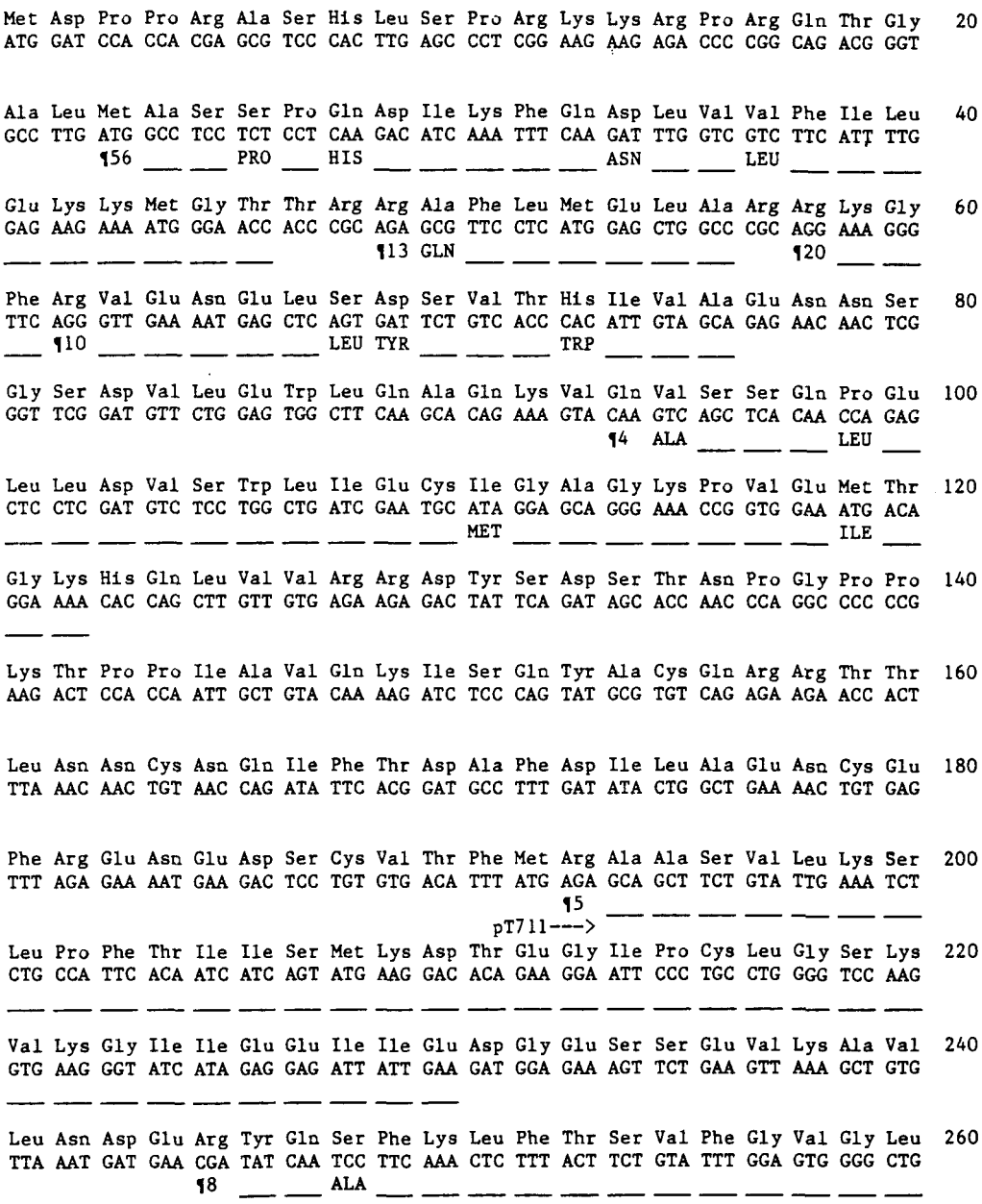

FIGURE 3. Complete coding sequence of human terminal transferase. Amino acid residues translated from nucleotides 329 to 1852 of the human terminal transferase cDNA, and the cDNA sequence are shown. Peptides from calf thymus terminal transferase (¶n) are shown with dashes at identical residues and mismatches spelled out. The N-terminal sequence of the 56 kDa peptide (¶56) and the 5′-ends of the pT711 and pT17 cDNA sequences are also indicated.

kDa peptide to 56 kDa would result in the loss of this signal and could account for the presence of the enzyme in the cytoplasm of cortical thymocytes. Thus, proteolysis might be a mechanism for regulating terminal transferase location in the cell.

The 58 kDa calf terminal transferase peptide can be phosphorylated with a cAMP dependent protein kinase while phosphorylation sites are absent in the 56 kDa peptide.[55] Analysis of phospho-amino acids produced by endogenous protein kinase in

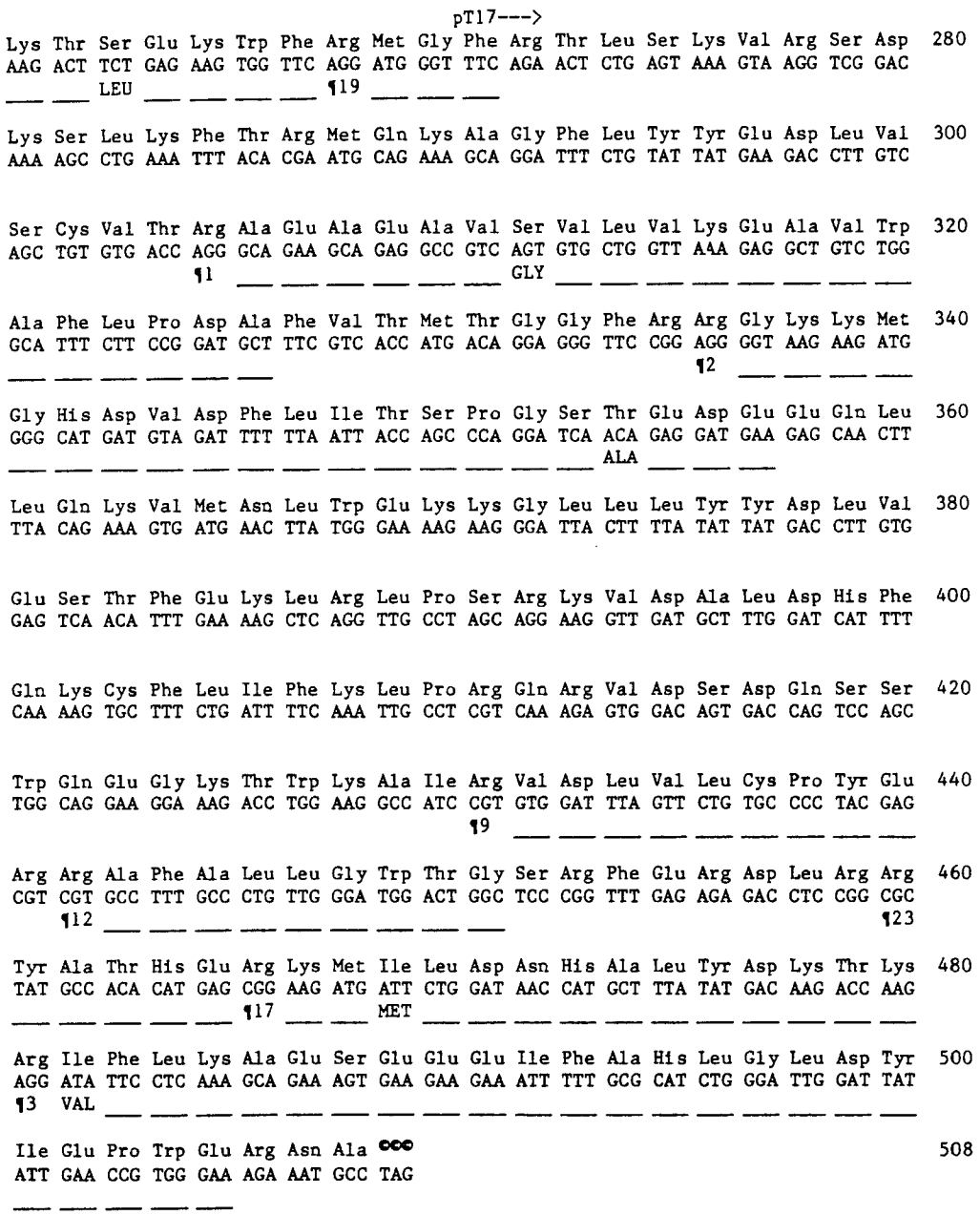

FIGURE 3 (continued)

terminal transferase from human lymphoblastoid cells showed 85% phosphoserine and 15% phosphothreonine.[56] These findings suggest to us that the phosphorylation site on terminal transferase must be located at a serine near the N-terminus of the protein.[58] The translated human terminal transferase sequence has the sequence -Arg-Ala-Ser-His-Leu-Ser- in residues 5 to 10, where Ser-7 could be phosphorylated and the sequence -Pro-Arg-Gln-Thr-Gly-Ala-Leu- at residues 16 to 22 where Thr could be the phosphorylation site. It is known that terminal transferase dissociates from chromatin prior to metaphase and reassociates after completion of mitosis.[17] Furthermore, induced differentiation of prelymphoid cells by phorbol ester results in the loss of terminal trans-

ferase and appearance of more mature lymphoid markers.[59-61] It is possible that phosphorylation of terminal transferase might play a role in localization, and thereby differentiation and/or regulation of its in vivo activity. We have not found any changes in deoxynucleotide polymerization activity induced by phosphorylation[55] under optimal reaction conditions.

Finally, it is possible to deduce a rough localization of the α- and β-peptides of the 32 kDa form of terminal transferase by analysis of the distribution of amino acids in the human cDNA sequence. We have previously shown that the bovine β-peptide contains five cysteines and the bovine α-peptide contains two cysteines.[45] In the human terminal transferase sequence, there are five closely grouped cysteines between amino acid 165 and 402, and two remaining cysteines between residues 403 to 508. This shows that the α-peptide must be located at the carboxyl end of the sequence and the β-peptide is N-terminal to the α-peptide. Similar conclusions have been drawn by comparison of amino acid sequences from α- and β-peptides derived from bovine terminal transferase with published partial cDNA sequence for the human enzyme.[62] These data suggest that the amino terminal 158 amino acids are unnecessary for the catalytic activity of terminal transferase. If the nuclear localization and phosphorylation signals are in the N-terminal 22 amino acids, then there is still a stretch of 136 amino acids in terminal transferase with no known function. The possibility that this domain of the protein contains biologically important activity remaining to be discovered is a subject for speculation and future investigation. It will also be of interest to see if the domain structure and sequence of this protein is consonant with the supergene family[63] associated with recognition molecules.

IV. MOLECULAR CLONING OF TERMINAL TRANSFERASE

The unique localization of terminal transferase in lymphoid cells undergoing differentiation stimulated a great deal of interest in isolation of the gene for this enzyme. Immunoprecipitation of in vivo synthesized terminal transferase protein and in vitro translation of terminal transferase mRNA from mice,[64,65] bovine,[65,66] and human[49] produced 55 to 60 kDa proteins. Our preliminary work on the fractionation of terminal transferase mRNA to obtain an enriched preparation for cloning purpose was disbanded because the mRNA is present in only trace amounts. Fractionation of total poly $(A)^+$-mRNA on formamide sucrose gradients demonstrated that terminal transferase mRNA from human lymphoblastoid cells sedimented at about 20 S. Development of the λgt 11 expression vector[67] made it feasible to attempt cloning of terminal transferase cDNA without enrichment by detection of expression using antibody screening. Two laboratories have been successful in cloning partial cDNA sequences for human and murine terminal transferase by this approach.

A. Cloning of Human and Murine Terminal Transferase cDNA

A partial human terminal transferase cDNA recombinant consisting of 939 base pairs was isolated from a lymphoblastoid cell (KM-3) cDNA library we prepared in λgt 11. This clone represented the sequence coding for about 60% of the carboxyl-terminus of the protein.[34] A partial murine terminal transferase cDNA was also isolated from a mouse EL-4 thymoma cDNA library in λgt 11 with a 1,400 base pair insert found to be the largest recombinant fragment.[68] This 1,400 bair pair sequence contains the poly (A) tail and should also represent the sequence coding for the carboxyl-end of terminal transferase protein, although no sequence information is available at this time. The cloned human terminal transferase cDNA fragment was proven to be terminal transferase sequence by comparison of translated sequence with determined amino acid sequences for the calf thymus terminal transferase and by translation of selected mes-

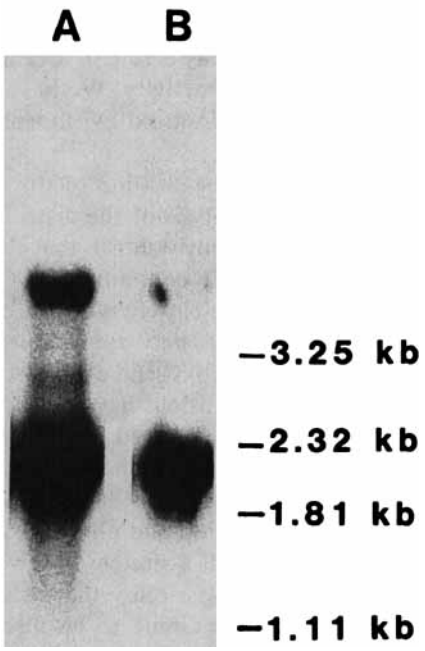

FIGURE 4. Blot hybridization of KM-3 RNA with cloned terminal transferase cDNA fragments. Poly (A)⁺-RNA from KM-3 cells was separated by electrophoresis on a 1.5% agarose gel containing 2.2 formamide in 0.2 *M* morpholinopropane-sulfonate at pH 7.0, 0.05 *M* sodium acetate and 5 m*M* EDTA, and transferred to a nitrocellulose sheet. Lane A shows the autoradiogram of mRNA hybridizing to nick-translated pT711 and lane B shows hybridization to pT106. Size markers used were restriction enzyme (Bgl I and Hinc II) fragments of pBR322 DNA.

sage. The partial human and murine cDNA sequences hybridize to a 2,000 to 2,200 nucleotide mRNA in terminal transferase containing cells and do not hybridize with any RNA in terminal transferase negative cell lines.[34,68]

The 939 base pair cDNA fragment was recloned into pUC-8 to give pT17 for DNA sequencing and to provide DNA probes to be used in the search for longer cDNA recombinants of terminal transferase. Rescreening of our λgt 11 libraries with the nick-translated insert of pT17 produced a cDNA clone for human terminal transferase containing a 2068 base pair insert.[35] This 2068 base pair insert contains the entire coding sequence for human terminal transferase in an open reading frame with a 328 nucleotide leader sequence and 202 nucleotides downstream from the termination codon. This 2068 nucleotide sequence was recloned as two Eco R1 fragments in pUC-8 sequencing plasmids to generate pT106 and pT711. Probing RNA blots of poly (A)⁺-mRNA from terminal transferase containing cells with nick-translated pT711 and pT106 demonstrated that both of these probes hybridize to an mRNA of about 2100 nucleotides (Figure 4). The entire DNA sequence for the 2068 base pair insert is presented in Figure 5.

Translation of the 2068 base pair sequence shows an open reading frame starting at nucleotide 329 and terminating at nucleotide 1852, producing a 508 amino acid residue protein with a calculated mol wt of 58,308 (Figure 3). The 2068 base pair insert of our clone does not represent the entire cDNA sequence for terminal transferase since the 5′-poly (A) sequence is not present. The presence of a stretch of 14 dG residues starting

```
          10         20         30         40         50         60
   1 TCATTGGGTG ATTGATTTCT ATGCTCCTTG GTGTGGACCT TGCCAGAATT TTGCTCCAGA
  61 ATTTGAGCTC TTGGCTAGGA TGATTAAAGG AAAAGTGAAA GCTGGAAAAG TAGACTGTCA
 121 GGCTTATGCT CAGACATGCC AGAAAGCTGG GATCAGGGCC TATCCAACTG TTAAGTTTTA
 181 TTTCTACGAA AGAGCAAAGA GAAATTTTCA AGAAGAGGGG GGGGGGGGGG CCCCCCCCAA
 241 AAACCCTTCG TGTAGGAGGG TGGCAGTCTC CCTCCCTTCT GGAGACACCA CCAGATGGGC

 301 CAGCCAGAGG CAGCAGCAGC CTCTTCCCAT GGATCCACCA CGAGCGTCCC ACTTGAGCCC
 361 TCGGAAGAAG AGACCCCGGC AGACGGGTGC CTTGATGGCC TCCTCTCCTC AAGACATCAA
 421 ATTTCAAGAT TTGGTCGTCT TCATTTTGGA GAAGAAAATG GGAACCACCC GCAGAGCGTT
 481 CCTCATGGAG CTGGCCCGCA GGAAAGGGTT CAGGGTTGAA AATGAGCTCA GTGATTCTGT
 541 CACCCACATT GTAGCAGAGA ACAACTCGGG TTCGGATGTT CTGGAGTGGC TTCAAGCACA

 601 GAAAGTACAA GTCAGCTCAC AACCAGAGCT CCTCGATGTC TCCTGGCTGA TCGAATGCAT
 661 AGGAGCAGGG AAACCGGTGG AAATGACAGG AAAACACCAG CTTGTTGTGA GAAGAGACTA
 721 TTCAGATAGC ACCAACCCAG GCCCCCCGAA GACTCCACCA ATTGCTGTAC AAAAGATCTC
 781 CCAGTATGCG TGTCAGAGAA GAACCACTTT AAACAACTGT AACCAGATAT TCACGGATGC
 841 CTTTGATATA CTGGCTGAAA ACTGTGAGTT TAGAGAAAAT GAAGACTCCT GTGTGACATT

 901 TATGAGAGCA GCTTCTGTAT TGAAATCTCT GCCATTCACA ATCATCAGTA TGAAGGACAC
 961 AGAAGGAATT CCCTGCCTGG GGTCCAAGGT GAAGGGTATC ATAGAGGAGA TTATTGAAGA
1021 TGGAGAAAGT TCTGAAGTTA AAGCTGTGTT AAATGATGAA CGATATCAAT CCTTCAAACT
1081 CTTTACTTCT GTATTTGGAG TGGGGCTGAA GACTTCTGAG AAGTGGTTCA GGATGGGTTT
1141 CAGAACTCTG AGTAAAGTAA GGTCGGACAA AAGCCTGAAA TTTACACGAA TGCAGAAAGC

1201 AGGATTTCTG TATTATGAAG ACCTTGTCAG CTGTGTGACC AGGGCAGAAG CAGAGGCCGT
1261 CAGTGTGCTG GTTAAAGAGG CTGTCTGGGC ATTTCTTCCG GATGCTTTCG TCACCATGAC
1321 AGGAGGGTTC CGGAGGGGTA AGAAGATGGG GCATGATGTA GATTTTTTAA TTACCAGCCC
1381 AGGATCAACA GAGGATGAAG AGCAACTTTT ACAGAAAGTG ATGAACTTAT GGGAAAAGAA
1441 GGGATTACTT TTATATTATG ACCTTGTGGA GTCAACATTT GAAAAGCTCA GGTTGCCTAG

1501 CAGGAAGGTT GATGCTTTGG ATCATTTTCA AAAGTGCTTT CTGATTTTCA AATTGCCTCG
1561 TCAAAGAGTG GACAGTGACC AGTCCAGCTG GCAGGAAGGA AAGACCTGGA AGGCCATCCG
1621 TGTGGATTTA GTTCTGTGCC CCTACGAGCG TCGTGCCTTT GCCCTGTTGG GATGGACTGG
1681 CTCCCGGTTT GAGAGAGACC TCCGGCGCTA TGCCACACAT GAGCGGAAGA TGATTCTGGA
1741 TAACCATGCT TTATATGACA AGACCAAGAG GATATTCCTC AAAGCAGAAA GTGAAGAAGA

1801 AATTTTTGCG CATCTGGGAT TGGATTATAT TGAACCGTGG GAAAGAAATG CCTAGGAAAG
1861 TGTTGTCAAC ATTTTTTCCT ATTCTTTTCA AGTTAAATAA ATTATGCTTC ATATTAGTAA
1921 AAGATGCCAT AGGAGAGTTT GGGGTTATTT AGGTCTTATT GAAATGCAGA TTGCTACTAG
1981 AAATAAATAA CTTTGGAAAC ATGGGAAGGT GCCACTGGTA ATGGGTAAGG TTCTAATAGG
2041 CCATGTTTAT GACTGTTGCA TAGAATTC
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FIGURE 5. Complete sequence of human terminal transferase cDNA. The start and stop codons are underlined.

at residue 217 followed by a stretch of 8 dC residues in this cDNA gave rise to the possibility that part of this leader sequence might be derived from the cloning procedures used and thus may not represent the true 5′-sequence of terminal transferase mRNA. This possibility was ruled out by demonstrating that a fragment containing residues 1 to 231 hybridizes to the same genomic DNA fragment as a fragment containing residues 232 to 342. Figure 6 shows a schematic diagram of the cDNA sequences coding for human terminal transferase.

B. Expression of Human Terminal Transferase in *Escherichia coli*

An unambiguous demonstration of the validity of coding sequence for a eukaryotic protein is provided if the protein can be expressed in *E. coli*. Production of human terminal transferase in *E. coli* was accomplished by inserting the coding sequence of human terminal transferase downstream from the lac Z promoter and in the same reading frame as the N-terminal codons of the lac Z gene carried by expression plasmid

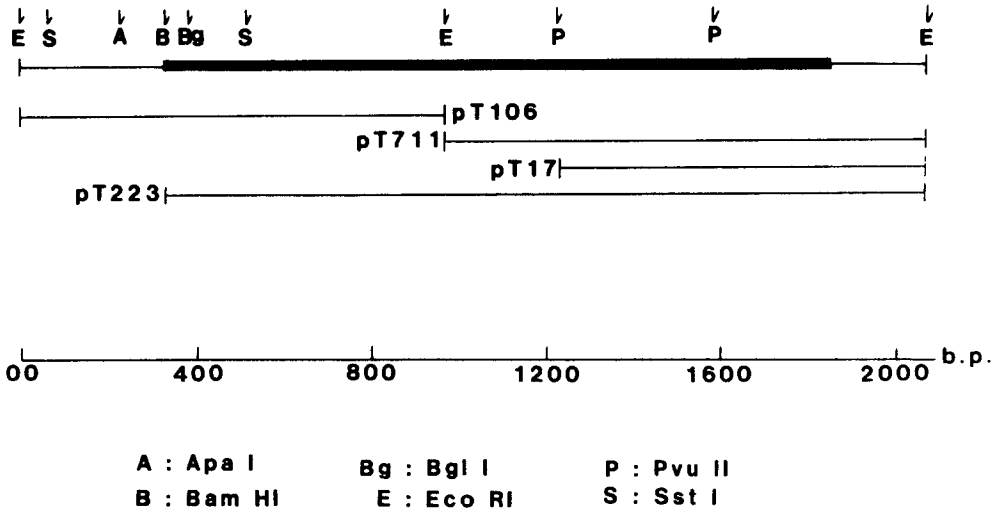

FIGURE 6. Schematic diagram of the cDNA sequence coding for human terminal transferase.

pUC-19.[35] When extracts of *E. coli* cells bearing this plasmid were made terminal transferase activity was indeed found. Activity is found in cells harboring the coding sequence in pT223 (Figure 3), with all sequences in correct orientation. No terminal transferase activity can be detected in extracts made from normal *E. coli* HB101 or *E. coli* HB101 carrying a control insert in pUC-19 with the carboxyl 60% of the coding sequence of terminal transferase in the opposite orientation. Immunoblotting of terminal transferase found in the active bacterial extract detected a protein band at around 60 kDa reacting with polyclonal antibody, consistent with a recombinant protein containing 15 nonterminal transferase amino acid residues at N-terminus followed by 507 terminal transferase residues.

The recombinant terminal transferase can be isolated directly from crude bacterial lysates using an immunoaffinity column made with mouse monoclonal antibody to human terminal transferase covalently linked to Protein A Sepharose. Using a one step procedure, terminal transferase present in the bacterial extract was purified to a specific activity of 25,000 units per milligram about 125-fold purification. Analysis of peptides in the immunoaffinity purified bacterial terminal transferase by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed three major stained peptides, 60 kDa, 58 kDa, and 54 kDa (Figure 7A, lane a). Immunoblot analysis showed that the 60 kDa and the 58 kDa peptides react with rabbit antibody to terminal transferase (Figure 7B, lane a), proving that the 60 kDa peptide is the recombinant terminal transferase. The 54 kDa peptide represents a contaminating peptide. Some degradation of the recombinant terminal transferase peptide is observed and a 58 kDa peptide is found to be the major degraded species (Figure 7B, land a). These results are an unequivocal demonstration that the cDNA sequence we have isolated represents human terminal transferase sequence.

Some uncertainty remains concerning the level of activity associated with different molecular species of terminal transferase. The homogeneous low mol wt (32 kDa) terminal transferase has been shown to have a specific activity of 100,000 dA units per milligram.[46] Purified 58 kDa form always has a turnover number several-fold lower than the 32 kDa form. It is possible that denaturation of the 58 kDa form during purification results in lower enzyme activity. It is also possible that the extra sequences present in the N-terminal half of the protein actually reduce dNTP polymerization activity. We have constructed plasmids of pT223 deleted from the N-terminal coding

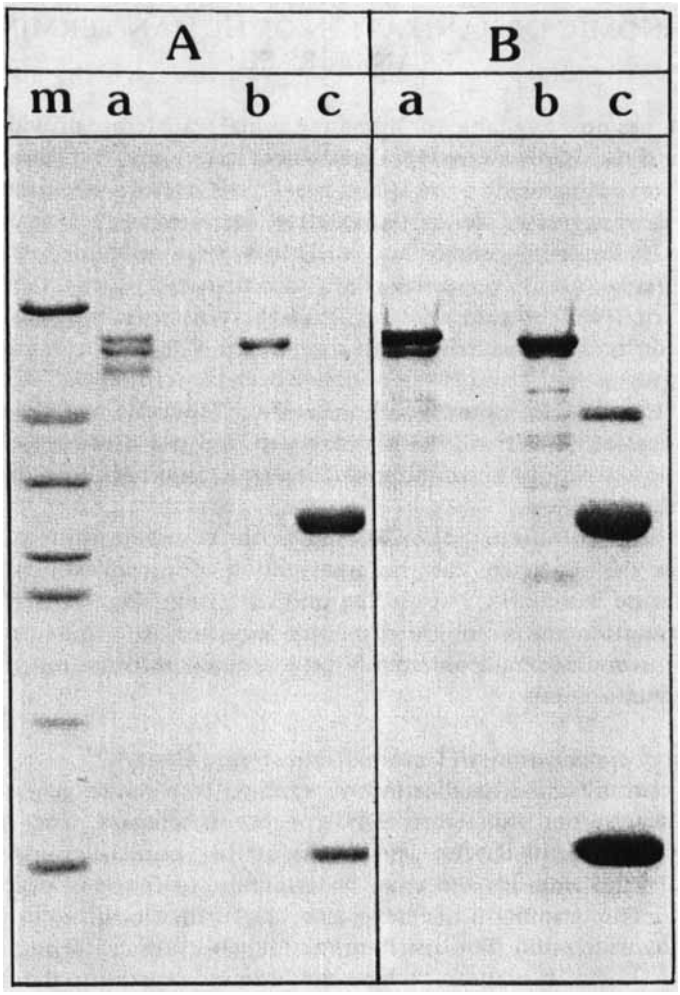

FIGURE 7. Gel analysis of recombinant human terminal transferase purified from *E. coli* extracts. Panel A shows the Coomassie Blue stained gel of purified *E. coli* terminal transferase and panel B shows the immunoblot of the same set of samples. Lane a represents 3 μg of immunoaffinity purified *E. coli* terminal transferase, lane b represents 2 μg of immunoaffinity purified human KM-3 cell terminal transferase, and lane c represents 5 μg of immunoaffinity purified low molecular weight calf thymus terminal transferase. Molecular weight markers (lane m) used were bovine serum albumin, ovalumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and α-lactalbumin.

sequences. Deletion of coding sequences from the N-terminus of the expression plasmid results in increased terminal transferase activity in the bacterial extracts. Extracts from *E. coli* cells carrying deletion plasmids of pT223 that code for only 352 to 360 amino acid residues of terminal transferase have activity threefold greater than extracts made from cells carrying full length pT223. These studies clearly show that the N-terminal amino acids are not required for the catalytic activity of terminal transferase, and the presence of these 160 additional amino acids may even reduce the absolute catalytic activity of the enzyme. The biological contribution of this protein domain remains to be determined.

V. GENOMIC ORGANIZATION OF HUMAN TERMINAL TRANSFERASE

The cDNA clones now available for human terminal transferase provide reagents for an examination of the chromosomal localization of the terminal transferase gene. Since terminal transferase is present in pre-B and pre-T cells during the period of immunoglobulin gene rearrangement, we are particularly interested in finding out if the gene for this enzyme is simple or complex and whether it might be located in or near members of the supergene family containing the histocompatibility locus, the immunoglobulin gene locus, or T-cell receptor clusters. Terminal transferase has been postulated to be responsible for inserting nucleotide sequences in the N-regions of immunoglobulin[12] and T-cell receptor genes;[8,9] and N regions have been detected at the junction between chromosomes 11 and 14 in chronic lymphocytic B-cell leukemia with the t(11;14) chromosome translocation.[69,70] It will be of interest to find out if this gene might play a role in oncogenic activation in hematopoietic malignancies that show aberrant expression of terminal transferase.

It is also possible to initiate molecular studies on the organization of the terminal transferase gene, genomic size, and the organization of intron/exon structure. Since terminal transferase is a marker, appearing only at certain stages of lymphoid differentiation, information concerning the promoter sequence of terminal transferase and control of this promoter could potentially generate new information concerning the regulation of hematopoiesis.

A. Chromosomal Localization of Terminal Transferase Gene

To find the chromosomal localization of terminal transferase gene, Isobe et al.[71] used our human terminal transferase cDNA probes to screen a panel of mouse and human somatic cell hybrid DNAs. The results of the Southern transfer analysis of hybrid DNAs demonstrate that the gene for terminal transferase is located on human chromosome 10. The terminal transferase gene was further localized to region 10q23->q25 by *in situ* hybridization. The distribution of labeled sites in 100 human metaphase chromosomes is shown in Figure 8, where the ordinate represents the number of labeled sites and the abscissa represents the chromosomes according to their relative size proportion. Of the 100 cells examined, about 20% of all grains were located on the long arm of chromosome 10 which represents approximately 3% of the haploid genome.

It thus appears that the gene for terminal transferase is not physically located within or near the histocompatibility, T-cell receptor or immunoglobulin loci. There are, however, some recent reports describing rearrangement of chromosome 10 in human T-cell neoplasms.[69,70] Analysis of these malignancies for rearrangements within or in proximity to the terminal transferase gene may provide a clue to the role of terminal transferase in human leukemogenesis.

B. Organization of the Human Terminal Transferase Gene

For preliminary analysis of the genomic structure of human terminal transferase, we used nick-translated inserts of pT711 and pT106 to probe human cell line DNAs after digestion with various restriction enzymes. The results of the Southern transfer analysis of human fibroblast DNA, pre- and mature lymphoid DNAs gave no indication of rearrangement of the terminal transferase gene.[72] The intensity seen in the hybridizations suggests that the terminal transferase gene is present in the cell in few copies, in agreement with the results of Landau et al.[68] Comparing the intensity of hybridization of genomic DNA fragments from various human cell lines showed that those from mature B (cell line RPMI-8392) and mature T cells (cell line RAMOS-1) are much

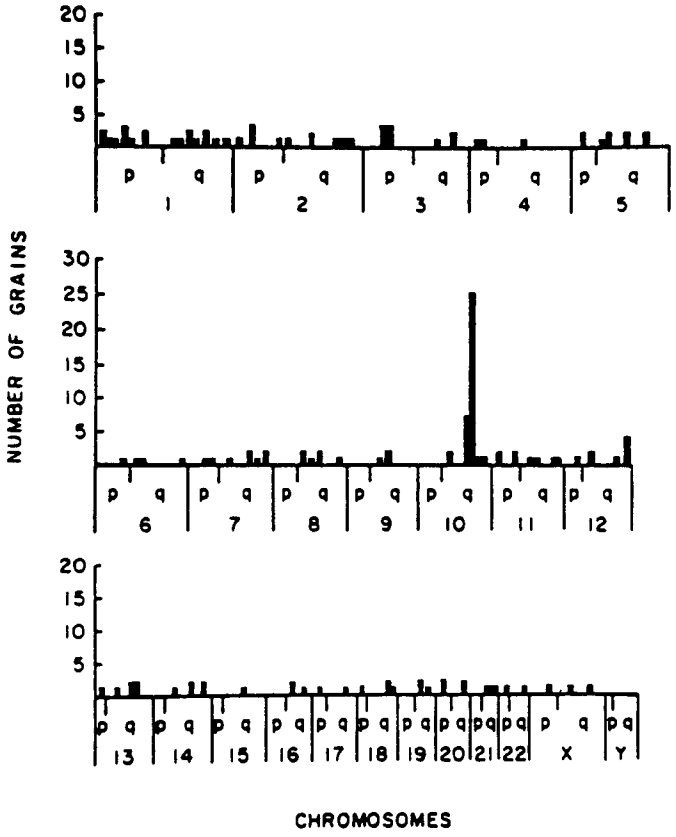

FIGURE 8. Localization of the terminal transferase gene on human chromosomes by *in situ* hybridization.

stronger than those seen with human fibroblast, pre-B (cell line RPMI-KM-3) and pre-T (cell line RPMI-8402) cells suggesting possible amplification of the terminal transferase gene during maturation of lymphoid cells. Since these lymphoblastoid cells are known to be aneuploid, detailed interpretation must await more appropriate biological materials. The Southern blot analysis does provide an estimate of the minimum size for the human terminal transferase gene as greater than 65 kb.[72] which is the sum of Bam H1 restriction fragments of the human fibroblast DNA hybridizing to the terminal transferase probe.

For detailed analysis of the genomic structure of human terminal transferase, we screened a human placenta DNA library constructed in Charon 28 phage with nick-translated inserts of pT106 and pT711. A total of 76 positive recombinant clones were isolated from 1.6×10^6 phages screened, of which 64 were found to have different inserts judging from restriction enzyme analysis. Of the 44 recombinant phages characterized, 17 showed possible rearrangements of the human sequences and so were not used for structural analysis. Of the remaining 27 recombinants, 8 hybridized with the nick-translated insert of pT711, 16 hybridized with nick-translated pT160, and 3 hybridized with both. These results provided a preliminary ordering of genomic fragments.

Southern transfer analysis of restriction fragments of human genomic DNA in Charon 28 using Pvu II fragments of the pT711 insert and Bam H1 fragments of pT106 (Figure 6) allowed further alignment of the genomic fragments along the terminal

transferase cDNA sequence. Ordering and alignment of the restriction enzyme sites by partial digestion and hybridization with the Charon 28 DNA fragments near the cloning site (Bam H1) should provide the restriction map as well as overlapping clones. Among the five sections of terminal transferase cDNA used for probes, we were able to find overlapping of genomic sequences only between two sections involving Eco R1 — Pvu II and Pvu II — Pvu II fragments. The total size of genomic fragments analyzed so far is greater than *120* kb, and we estimate that the complete size of the human terminal transferase gene may be twice this size. Further analysis requires rescreening the human placenta DNA library for additional recombinants and/or larger genomic DNA fragments in other genomic libraries. The restriction enzyme sites of the genomic fragments of human terminal transferase are presented on the partial map of Figure 9.

It is of interest to note that we do have a large number of recombinants containing inserts hybridizing with the Eco R1 — Apa I fragment that contains the 230 bp cDNA sequence corresponding to the 5′-end of terminal transferase mRNA. Analysis of the upstream sequences in the genomic fragments should provide the promoter sequence for this enzyme and possibly for other lymphoid differentiation markers. The current state of our analysis of the terminal transferase gene shows that it is a single copy nonrearranged structure on human chromosome 10.

VI. SOME THOUGHTS ABOUT TERMINAL TRANSFERASE ACTION

A. The Functional Entities of Humoral and Cellular Immunity

Immunoglobulin genes are constructed in several parts. Variable region sequences, D-regions, and J-regions are distributed over wide regions of the chromosome and are separated from the multiple C-region sequences.[73] The total DNA sequence encoding heavy chain immunoglobulin sequences probably spans thousands of kilobases on chromosome 14 in human tissues that have not rearranged them for immunoglobulin synthesis. During the process of differentiation in B-cells, a V-region is selected and brought into recombination with a selected D-region, and then further recombined with a J-region to form a "V-gene".[74,75] The initial selection from $C\mu$ sequences is made for recombination with the V-gene and further recombination with one of several $C\gamma$ sequences (or other heavy chain classes) occurs at the time of class switching. This may result in abrogation of total DNA in the differentiating chromosome to produce the final rearranged sequence, but the exact loss is not clear.[76] The rearranged sequence still contains the normal intron/exon structure characteristic of eukaryotic genes, but now the total immunoglobulin sequence to be transcribed has been reduced by several orders of magnitude. The rearranged gene is transcribed to RNA, matured to a message of around 2 Kb and then translated to heavy chain protein.[77]

So far this description of immunoglobulin gene rearrangement uses only those sequences present in germ-line DNA, but additional diversity occurs at the recombinational joints on either end of the D (for divesity) segment.[74] These joints form the major hypervariable regions in the heavy chain of immunoglobulin molecules. Similar recombining regions occur in light chains producing total combinational possibilities exceeding 10^7, although some possible recombinations could produce out of phase sequences. At the joining regions even greater diversity is observed. Part of this diversity involves short additional sequence on either side of the D region that cannot be found in germ line DNA. These regions have been called N-regions[7] or NGEs,[78] for nongerm-line elements. They must be placed in immunoglobulin DNA sequence by some form of creative DNA polymerase acting during the recombinational events associated with differentiation of B-cell chromosomes.

The structure of the T-cell receptor genes have only recently been elucidated[79-83] and is still in a somewhat fluid state. The current concept of structure is quite analogous to

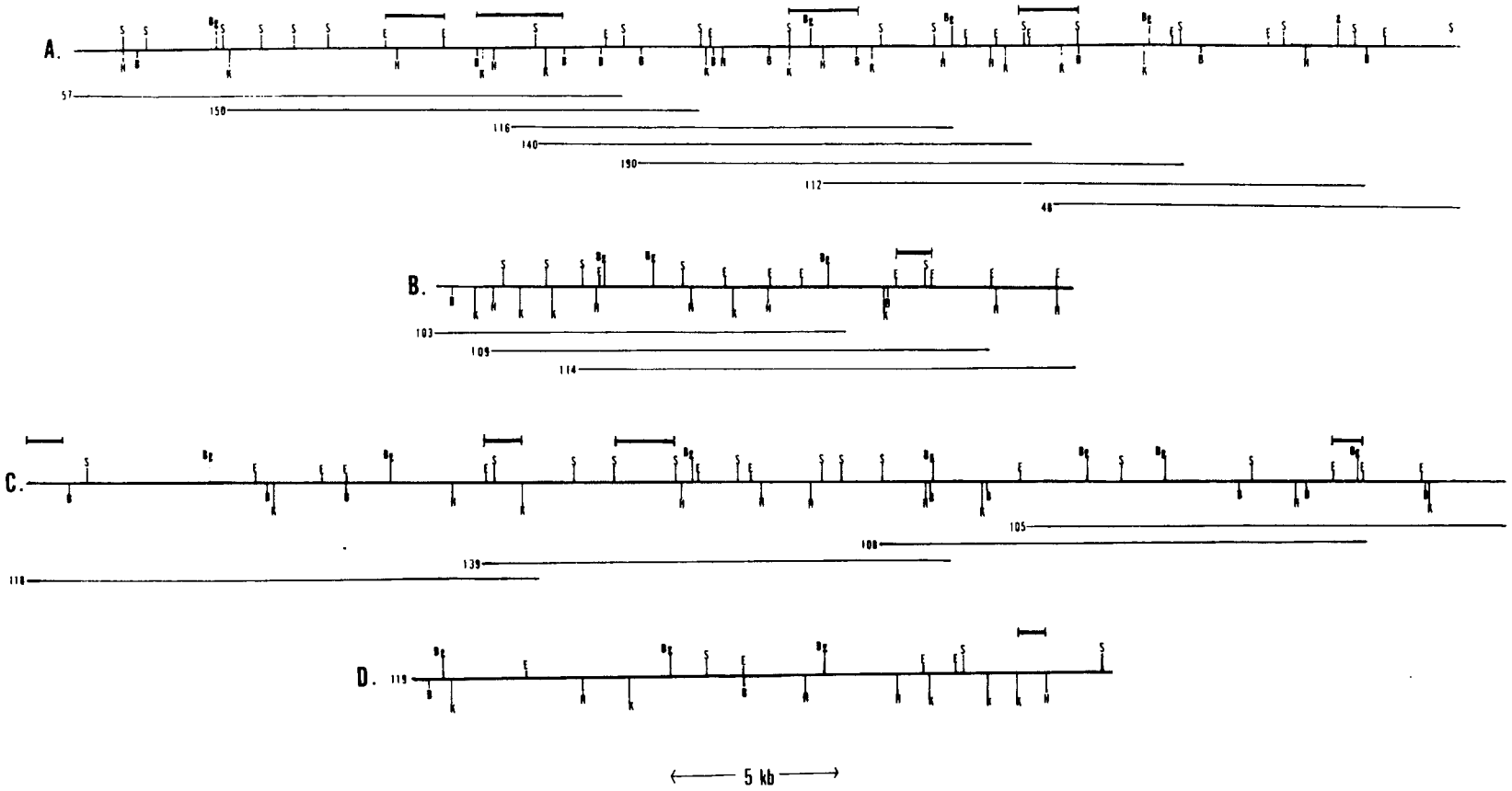

FIGURE 9. Partial structure of human terminal transferase gene. The results of 14 of the 27 recombinant phages from human placenta DNA in Charon 28 phage analyzed by hybridization and partial restriction analysis are presented. The insert sizes range from 11 kb to 22 kb. The restriction sites were aligned by partial restriction enzyme digests probed with fragments from the arms of Charon 28 phage. Recombinant phages in group A hybridize with Eco R1-Apa I and Apa I-Bam H1 fragments of the insert in pT106, phages in group B hybridize with the Bam H1-Eco R1 fragment of the insert in pT106, phages in group C hybridize with Eco R1-Pvu II and Pvu II-Pvu II fragments of the insert in pT711, and the phage in group D hybridizes with the Pvu II-Eco R1 fragment of the insert in pT711 (corresponding to the 3'-end of terminal transferase mRNA). Bars above each group indicate the fragments hybridizing with terminal transferase probes.

that found in immunoglobulins, but with only one variable region domain, and bearing considerable homology to immunoglobulin structure as well as sequence. Most notably the T-cell receptor genes undergo chromosomal rearrangements quite comparable to the V and C joining in immunoglobulin genes. N-regions are also found in T-cell receptor joints, completing the analogy between humoral and cellular immune systems.[8,9] Several other recognition proteins are also members of this supergene family.[63] Many details remain to be discovered but the similarity seems ripe for sweeping generality.

B. The Enzymes Available for DNA Diversification

No detailed molecular mechanisms for the recombinational processes occurring during immunoglobulin gene or T-cell receptor gene maturation are available for discussion although some rules for recombination have been demonstrated.[63] Quite a bit is known about eukaryotic enzymes that can polymerize deoxynucleotides. Eukaryotic DNA polymerases include DNA polymerase-α, -β, -γ, and -δ. None of these enzymes are restricted in organ localization. All have been described as replicative enzymes, requiring template direction as well as some suitable initiation point for chain growth. The only enzyme known to polymerize deoxynucleotides without template direction is terminal deoxynucleotidyl transferase. It is the only enzyme known to have the properties requisite for *de novo* addition of deoxynucleotides at the discontinuities in DNA molecules that must occur during recombinational processes. It is also significant that terminal transferase occurs only in primary lymphoid organs where differentiation to diversification is occurring. Terminal transferase occurs in those organs and at those times when recombinational events leading to immunoglobulin and T-cell receptor diversification is occurring. Terminal transferase is the only relevant protein known to occur in pre-T and pre-B cells. In almost 30 years of research on eukaryotic DNA polymerases, terminal transferase is the only DNA polymerase found to fulfill the qualifications for DNA diversification in immunological systems.

We believe that terminal transferase alone is responsible for the somatic diversification in N-regions of immunity molecules. Presently available evidence for the participation of terminal transferase in DNA diversification in biological systems is largely circumstantial,[12] and somewhat subjective. But new clues continue to accumulate. Purification of terminal transferase on monoclonal immunoaffinity columns[35,54] has not produced any form of complex involving other proteins; so far only the 58 kDa species has been found. It would be of interest to examine this fractionation more closely to see if any evidence for possible complexes can be gained. If other proteins do not facilitate terminal transferase action then the total biological function must reside within the 508 amino acid sequence. The catalytic site for nucleotide polymeriziation is in residues 160 to 508 of the protein. Residues 1 to 159 could contain regions of importance for the biological function of terminal transferase. We have postulated a nuclear localization site within residues 1 to 23, and have suggested that this site is controlled by cAMP phosphorylation. We also think that additional important DNA-binding and possibly other catalytic activities might reside in the N-terminal sequence.

The minimum model for a diversification system is that it must contain a recognition site for immunoglobulin and T-cell receptor DNA recombination sites as well as the ability to add nucleotides. If N-region synthesis does not occur at pre-existing breaks in DNA chains, then the terminal transferase molecule might also have the properties of a single stranded restriction enzyme, producing the breaks necessary for nucleotide addition. The essential conjecture is that terminal transferase acting alone or in concert with other proteins must make additions at breaks induced by the recombination process or at sequence specific breaks induced by the enzyme itself.

These hypotheses can now be tested with appropriate biological and genetically engineered systems. The factors controlling the activity of this enzyme still remain un-

known, but an examination of the action of terminal transferase in DNA diversification is now an experimental possibility. This should stimulate further research into the relative contribution of the several regions of diversity to immunological competence and how they are subject to environmental signals. Perhaps this will provide new insight into the depth of immunological reactivity at the molecular level and levitate new immunological theories.

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