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Immunology

Cloning of terminal transferase cDNA by antibody screening

(λgt11/pre-B-lymphoid cells)

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ABSTRACT **A cDNA library was prepared from a terminal deoxynucleotidyltransferase-containing thymoma in the λ phage vector λgt11. By screening plaques with anti-terminal transferase antibody, positive clones were identified of which some had β-galactosidase-cDNA fusion proteins identifiable after electrophoretic fractionation by immunoblotting with anti-terminal transferase antibody. The predominant class of cross-hybridizing clones was determined to represent cDNA for terminal transferase by showing that one representative clone hybridized to a 2200-nucleotide mRNA in close-matched enzyme-positive but not to enzyme-negative cells and that the cDNA selected a mRNA that translated to give a protein of the size and antigenic characteristics of terminal transferase. Only a small amount of genomic DNA hybridized to the longest available clone, indicating that the sequence is virtually unique in the mouse genome.**

Terminal deoxynucleotidyltransferase (TdT) is a unique DNA polymerase that without template direction catalyzes the addition of deoxyribonucleotides onto the 3′-hydroxyl ends of DNA primers (1–3). It is present in the immature fraction of thymocytes (4–7), in a small fraction of bone marrow cells (5, 8), in transformed pre-B- and pre-T-cell lines (9, 10), and in leukemic cells (6, 11). The enzyme purified by the method of Yoneda and Bollum (12) and Chang and Bollum (13) is a dimer of M_r 26,000 and M_r 8,000 chains. This structure is, however, an artifactual result of proteolytic cleavage during the purification. The enzyme is synthesized as a single chain of M_r 58,000 in mice and M_r 55,000–60,000 in humans (14–18).

The function of TdT is not fully established. It was suggested that the enzyme might be responsible for somatic point mutation of immunoglobulin genes (19), but it now appears that somatic point mutation occurs late in B-lymphocyte maturation, when cells no longer contain TdT (20, 21). A recent proposal that TdT might be responsible for inserting nucleotides (*N* regions) at V_H–*D* and *D*–J_H (22) junctions (junctions of heavy chain variable–diversity and diversity–joining region genes) has received experimental support (unpublished results). *N*-region insertion in a T-cell receptor chain may also occur (23) and is possibly a result of the relatively high TdT levels in thymocytes.

We describe here the isolation from a thymoma cell line cDNA library of a clone that encodes TdT. Because the amino acid sequence of TdT has not been reported, there was no nucleic acid probe available for screening recombinants. Instead, specific antibodies raised against the purified two-chain bovine enzyme, which cross-react with the murine enzyme, were used to probe a cDNA library constructed in the λgt11 expression system of Young and Davis (24). As well as providing a cDNA clone with which to characterize TdT, this work shows the feasibility of cloning cDNA representations of rare mRNAs with conventional antibody reagents.

METHODS

λgt11 cDNA Library Construction. Double-stranded cDNA was synthesized from 20 μg of RLδ11 poly(A)-containing RNA, protected by reaction with *Eco*RI methylase, treated with S1 nuclease to produce blunt ends, and ligated to *Eco*RI linkers as described (25). The cDNA was digested with *Eco*RI and purified by chromatography on Bio-Gel A15M in TEN buffer (10 mM Tris, pH 8.0/1 mM EDTA/0.15 M NaCl). It was then ligated to *Eco*RI-cut λgt10 DNA, packaged, and grown on BNN102, an *hfl* strain of *Escherichia coli* in which phages that lack a cDNA insert are lysogenized (24). Phage that contain cDNA inserts can be recognized by their clear plaques on BNN102 (25). DNA was purified from a pool of 3×10^6 independent phage clones, 97% of which contained inserts as judged by their clear plaque morphology. The cDNA was transferred to the λgt11 vector by inactivating the λgt10 recombinant DNA by briefly digesting with BAL-31 exonuclease, digesting with *Eco*RI, and ligating to *Eco*RI-cleaved, phosphatase-treated λgt11 DNA.

Phage DNA was then packaged and plated on Y1088 (24), a *laci*^{q} strain of *E. coli* that represses transcription of the β-galactosidase-cDNA fusion protein. From the resulting phage, 10^6 were plated on KM392 *E. coli*, and the plaques were transferred to nitrocellulose filters (Schleicher & Schuell; BA85 0.45 m, 1322 mm).

Antibody Screening of Phage Library and Protein Blotting. Filters were washed in phosphate-buffered saline/1% bovine serum albumin/0.1% Nonidet P-40 (NP-40) and probed by the application of phosphate-buffered saline/1% albumin/0.1% NP-40 containing rabbit anti-calf terminal transferase affinity-purified antibody (P-L Biochemicals; 0.5 μg/ml) for 12 hr at 4°C. After washing, the filters were gently agitated in albumin/NP-40 containing 0.2×10^6 cpm of ^{125}I-labeled *Staphylococcus aureus* protein A (^{125}I-protein A) per ml for 2 hr at 4°C. The filters were washed extensively in albumin/NP-40 at 4°C and exposed to x-ray film for 2 days. Positive clones were picked from the master plates and purified through two additional rounds of screening. Several of the phage inserts were recloned into the *Eco*RI site of pBR322 (producing the plasmid used most extensively, pTdT20).

To examine the β-galactosidase-cDNA fusion proteins for immunoreactivity, phage lysogens were derived (24). Lysogens were induced at 45°C with or without 2 mM isopropyl-β-D-thiogalactopyranoside (iPrSGal*p*) for 2 hr. Bacteria were lysed in reducing sample buffer, and the proteins were sepa-

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Abbreviations: TdT, terminal deoxynucleotidyltransferase; bp, base pair(s); iPrSGal*p*, isopropyl-β-D-thiogalactopyranoside.

rated by electrophoresis through 7% $NaDodSO_4$/polyacrylamide gels. The separated proteins were transferred to a nitrocellulose filter (26) and probed by treatment with 0.5 μg of antibody per ml and 1×10^7 cpm of ^{125}I-protein A as described above for antibody screening.

RNA and DNA Filter Hybridization. RNA was prepared from cells or tissue by the guanidinium thiocyanate procedure (27) and selected for poly(A)-containing RNA by using an oligo(dT)-cellulose column (Collaborative Research, Waltham, MA; type III). From the poly(A)-selected RNA, 10 μg was denatured by heating to 56°C in 2.2 M formaldehyde/50% formamide and fractionated by electrophoresis through 1.2% agarose containing 2.2 M formaldehyde. The fractionated RNA was transferred to nitrocellulose and hybridized to ^{32}P-labeled pTdT20 as described (28).

Genomic DNA (10 μg) was cleaved with restriction enzyme and fractionated by electrophoresis through 1.0% agarose. Cloned phage DNA (2 μg) was cleaved and fractionated on 1.4% agarose. Transfer of the DNA to nitrocellulose and hybridization with nick-translated plasmid DNA was as described (28). Genomic DNA was probed with the purified cDNA insert from plasmid pOK6, a 1400-base-pair (bp) cDNA-containing plasmid derived by screening an EL4 thymoma cell line cDNA library (29) with the pTdT20 probe. Filters were exposed with an intensifying screen at −70°C.

***In Vitro* Translation and Hybrid Selection.** From the plasmid DNA, 10 μg was spotted onto nitrocellulose filters, denatured by exposure to 0.5 M NaOH for 7 min, neutralized in 0.1 M Tris (pH 7.4) followed by 0.15 M Na citrate/1.5 M NaCl, pH 7.4, for 10 min, and baked *in vacuo* for 1 hr. Poly(A)-containing RNA (266 μg/ml) from RL♂11 was hybridized to the filters in 50% formamide/20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)/0.2% $NaDodSO_4$/0.6 M NaCl/0.1 mg of tRNA per ml at 70°C for 10 min followed by 4 hr at 42°C. The filters were washed in 10 mM Tris, pH 7.6/0.15 M NaCl/1 mM EDTA/0.5% $NaDodSO_4$ at 65°C, and the RNA was eluted in H_2O containing 0.1 μg of tRNA per ml at 100°C for 1 min. The eluted RNA was ethanol-precipitated, lyophilized, and dissolved in 10 μl of H_2O. The RNA was translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories) in the presence of 1 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per μl. Translated products were immunoprecipitated with 2 μl of antibody or normal rabbit serum in buffer containing 10 mM sodium phosphate (pH 7.5), 100 mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, and 0.1% $NaDodSO_4$. Immunocomplexes were collected on *S. aureus*, eluted by boiling in gel buffer containing 3% 2-mercaptoethanol, and separated by electrophoresis through 7–20% gradient $NaDodSO_4$/polyacrylamide gels.

The anti-TdT antiserum was prepared by injection of two rabbits with TdT extracted from calf thymus and purified through the hydroxylapatite step (13, 14). RL♀3 cells (30) were metabolically labeled as described (14), and lysates were immunoprecipitated as described above.

RESULTS

The radiation-induced BALB/c thymoma cell line RL♂11 stably synthesizes TdT (14, 31) and, therefore, was used as a source of mRNA for the synthesis of a cDNA library. The cDNA was cloned initially into the *Eco*RI site of λgt10 because of this phage's high cloning efficiency (unpublished observations) and then was transferred into the *Eco*RI site of λgt11. Because the *Eco*RI site of λgt11 lies in the β-galactosidase gene, the presence of a cDNA insert causes loss of the blue-plaque phenotype when the phage is grown on *lacZ*$^-$ bacteria and plated on medium containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (24). Of the λgt11 plaques, 93% were colorless and therefore contained inserts. Screening of 10^6 independent phage clones with affinity-purified anti-TdT antibody and ^{125}I-protein A resulted in 25 positive clones (0.0025%).

To demonstrate that the reactivity of the phage clones with the antibody probe was due to antigenic determinants on the cDNA translation product, four of the clones were tested to determine if the β-galactosidase-cDNA fusion protein bound the antibody. Such a protein is larger than most other *E. coli* proteins and is readily visible after electrophoretic separation of the lysogen proteins. The proteins were visualized by transferring to nitrocellulose paper and probing with anti-TdT antibody followed by treatment with ^{125}I-protein A. For this experiment, protein lysates from lysogens induced by temperature shift and either induced or uninduced with iPrSGal*p* were analyzed. Temperature shift inactivates the c1857 λ repressor, causing replication of the provirus and partial derepression of the β-galactosidase-cDNA fusion protein. Addition of iPrSGal*p* fully derepresses the system, allowing maximal fusion protein synthesis. The wild-type λgt11 lysogen showed no specific bands with or without iPrSGal*p* (Fig. 1, lane gt) even though the major protein in iPrSGal*p*-induced lysates was β-galactosidase (not shown). Clone 3 and clone 12 showed a specific band migrating slightly slower than β-galactosidase. Clone 18 and clone 20 showed no specific bands. The absence of a specific band in clone 20 is surprising because, as shown below and discussed later, the cDNA in this clone overlapped in sequence with that of clone 3. These results show that in at least two of four cases, the antibody recognized the cDNA translation product and not a phage or bacterial protein.

Six of the clones giving the strongest signals with the antibody probe were compared to each other for nucleic acid sequence homology. The cDNA insert from λTdT20 was cloned into pBR322, and the plasmid, pTdT20, was used to

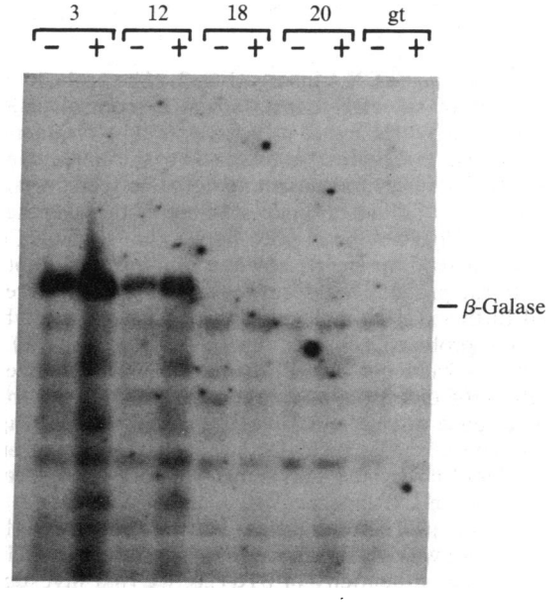

FIG. 1. Specific binding of anti-TdT antibody to the β-galactosidase-cDNA fusion protein synthesized by λgt11 clones indicated at the top of the lanes. λgt11 clones detected by antibody screening were lysogenized in *lacI*^{q} *E. coli*. Lysogens were grown at 37°C, shifted to 45°C, and then uninduced (−) or induced (+) with iPrSGal*p*. Lysogen proteins were fractionated by electrophoresis through 7% $NaDodSO_4$/polyacrylamide gels, transferred to nitrocellulose, and hybridized to anti-TdT antibody followed by treatment with ^{125}I-protein-A. Exposure was for 12 hr at −70°C with an intensifying screen. Lysogens shown above were constructed from phage clones 3, 12, 18, and 20 and from wild-type λgt11 (lane gt). The position of β-galactosidase (β-Galase) was determined from the mobility of purified β-galactosidase on an identical gel stained with Coomassie blue.

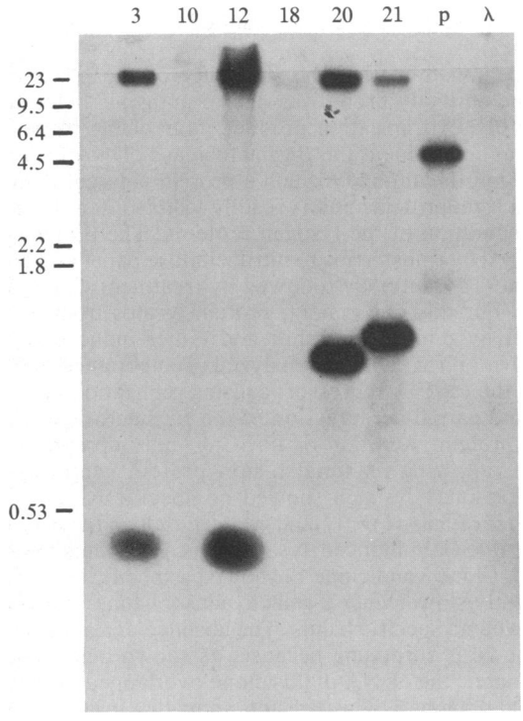

FIG. 2. Hybridization of pTdT20 to cDNA inserts of λgt11 clones detected by antibody screening. DNA from phage clones was digested with restriction enzyme *Eco*RI to excise the cDNA inserts, separated by electrophoresis on 1.4% agarose, and transferred to nitrocellulose. The filter was hybridized to ^{32}P-labeled pTdT20, a plasmid which contains the cDNA insert of phage 20 cloned into the *Eco*RI site of pBR322. Lanes: phage clones 3, 10, 12, 18, 20, and 21, linearized pBR322 (lane p), and *Hin*dIII-cleaved phage λ DNA (lane λ).

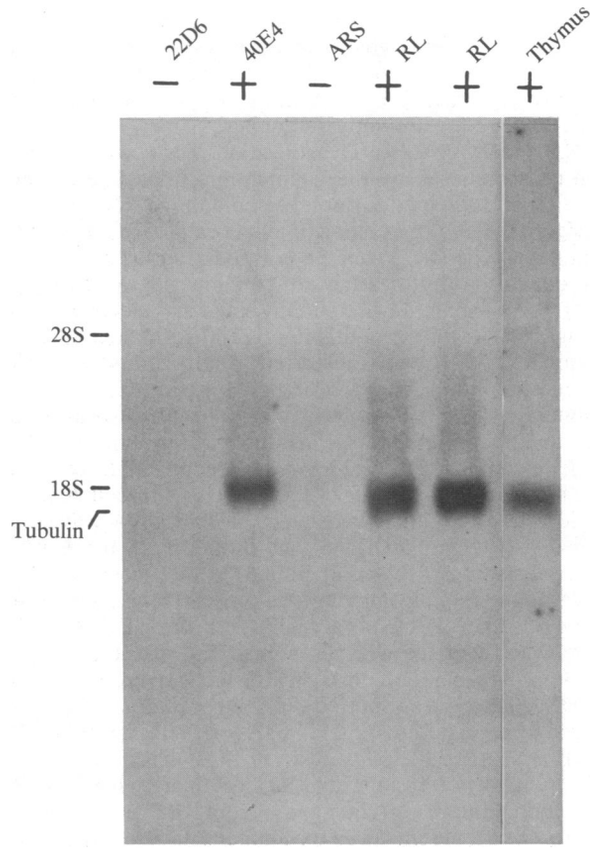

FIG. 3. Differential expression of mRNA hybridizing to pTdT20 in mouse cell lines and thymus tissue. Poly(A)-containing RNA was separated by electrophoresis through 1.2% agarose in the presence of formaldehyde. The RNA was transferred to nitrocellulose and probed with ^{32}P-labeled pTdT20. Size markers were 28S and 18S ribosomal RNA and tubulin mRNA (1800 bp) detected by subsequent hybridization to a rat tubulin cDNA probe. The presence (+) or absence (−) of TdT in cells as assayed by immunoprecipitation and enzymatic activity is indicated. Lanes: 22D6 and 40E4, Abelson murine leukemia virus-transformed pre-B-cell lines 22D6 (10 μg) and 40E4 (10 μg); ARS, B-cell hybridoma ARS (10 μg); RL (in order), BALB/c thymoma RL♂11 (6 μg) and RL♂11 (10 μg); and BALB/c thymus (6 μg).

probe the six phage cDNA inserts (Fig. 2). The probe hybridized to four of six cDNA inserts and to control pBR322 DNA. pTdT20 hybridized to its cognate 950-bp fragment, to the 450-bp insert of clones 3 and 12, and to the 1000-bp insert of clone 21. No insert from clone 10 could be seen on examination of the ethidium bromide staining of the agarose gel (not shown). Phage clone 21 grew poorly (not shown). Clone 18 showed a 1600-bp insert; however, its insert did not hybridize to the probe. Therefore, these three clones were not studied further. (High molecular weight bands that hybridized to the probe represented uncleaved phage DNA.) The insert bands were not derived from phage DNA since the pTdT20 probe did not hybridize to λ phage DNA, as shown by the absence of bands in *Hin*dIII-cleaved λ DNA (Fig. 2, lane λ). These results indicate that at least four out of six clones identified by the polyclonal antibody were derived from a single mRNA species.

Because the pTdT20 clone was identified only by antibody binding, there was no assurance that it represented TdT. To determine the authenticity of pTdT20, we first investigated the correlation of the presence of mRNA hybridizing to the clone with TdT enzyme and protein levels in various cell lines and in thymus tissue. Both RL♂11 cells and thymus, which express TdT protein as determined by both immunoprecipitation and enzymatic activity (4, 14), contained a single 2200-nucleotide mRNA that hybridized to the probe (Fig. 3). In contrast, two cell types that lack TdT, fibroblasts (L cells; not shown) and myelomas (represented by the ARS hybridoma in Fig. 3), showed no detectable pTdT20-related mRNA even after 10-fold longer exposure of the autoradiogram. The most telling comparison was between two Abelson murine leukemia virus-transformed cell lines that rearrange their heavy chain loci and, therefore, are at the same stage of pre-B-lymphocyte differentiation (32). Cell line 40E4 expressed TdT as assayed in both enzymatic activity and immunoprecipitation with anti-TdT antibody; the other cell line, 22D6, was negative by both assays (unpublished data). The 40E4 cell line expressed the 2200-nucleotide TdT mRNA, while the 22D6 cell line did not. Because the two transformants are nearly identical in their stage of differentiation, the correlation of enzyme level with the 2200-nucleotide mRNA strongly supports the identity of the cDNA clone as TdT.

To demonstrate that the nucleotide sequence of pTdT20 is complementary to that of TdT mRNA, hybrid selection of mRNA using the plasmid DNA was employed. To show the electrophoretic mobility of *in vivo* synthesized TdT, cells from the mouse thymoma cell line RL♀3 were metabolically labeled with [^{35}S]methionine. Lysates of the labeled cells were immunoprecipitated with normal rabbit serum or anti-TdT antibody (Fig. 4, lanes 1 and 2). A protein of M_r 58,000 was specifically immunoprecipitated by the anti-TdT antibody. It has been shown previously that such an antiserum precipitates a protein of this size that has TdT enzymatic activity and corresponds to the full-length protein (14). The specifically immunoprecipitated *in vitro* translation product (Fig. 4, lanes 3 and 4) comigrated with the protein specifical-

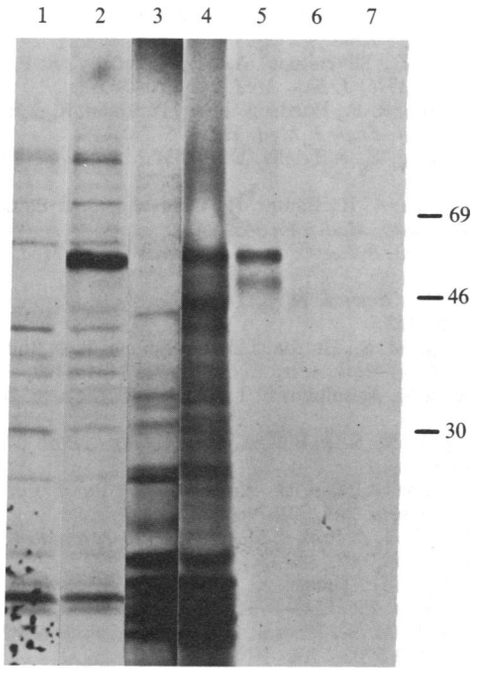

FIG. 4. Immunoprecipitation with anti-TdT antibody of *in vitro* translated protein products from RL♂11 mRNA specifically hybridizing to pTdT20. Lysates of [^{35}S]methionine-labeled proteins from RL♀3 cells were immunoprecipitated with normal rabbit serum or anti-TdT antibody (lanes 1 and 2). Poly(A)-containing RNA from RL♂11 cells was translated in a reticulocyte lysate system and immunoprecipitated with normal rabbit serum or anti-TdT antibody (lanes 3 and 4). RL♂11 RNA was hybridized to filter-bound pTdT20, pBR322, or a rat tubulin cDNA-containing plasmid (lanes 5, 6, and 7, respectively). Bound RNA was eluted and translated, and the protein products were then immunoprecipitated with anti-TdT antibody.

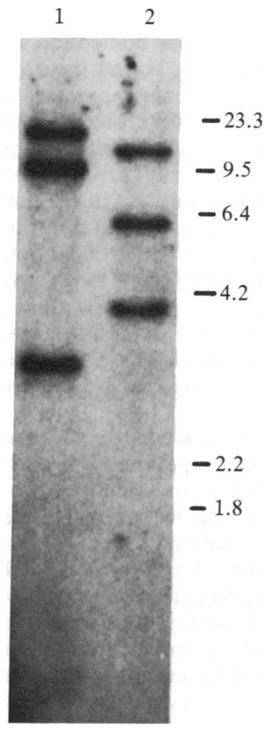

FIG. 5. Mouse genomic DNA probed with pOK6. DNA isolated from C57Bl/6 thymoma cell line EL4 (10 μg) was digested to completion with restriction enzymes *Bam*HI (lane 1) or *Pst* I (lane 2), fractionated by electrophoresis through 1.0% agarose, and transferred to nitrocellulose. The filter was hybridized to the TdT cDNA clone, pOK6. X-ray film was exposed for 2 days at −70°C with an intensifying screen.

ly immunoprecipitated from metabolically labeled thymoma cells. When RL♂11 RNA was first hybridized to filters to which was bound pTdT20 DNA, eluted, and then translated, the only translation product found that was not in the control sample was the M_r 58,000 band (Fig. 4, lane 5). By contrast, when a tubulin cDNA plasmid or pBR322 DNA was bound to the filter and similarly processed, no specific band was detectable (Fig. 4, lanes 6 and 7). Therefore, the plasmid pTdT20 specifically hybridizes to an mRNA that directs the translation of TdT protein. To determine the approximate amount of DNA in the mouse genome that could hybridize to the TdT cDNA clone, mouse liver DNA was cleaved with the infrequent cutting restriction enzymes *Bam*HI or *Pst* I, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose. To detect as much TdT-encoding genomic DNA as possible, a longer TdT cDNA clone was obtained by screening a cDNA library constructed from the thymoma cell line EL4, with a pTdT20 probe. This cDNA clone, pOK6, contains a 1400-bp insert that begins at the 3′ poly(A) tail of the mRNA and encompasses the entire nucleotide sequence of the pTdT20 insert (not shown). The cDNA insert from pOK6 was labeled by nick-translation and hybridized to the filter (Fig. 5). An autoradiogram of the filter showed, with *Bam*HI digestion, bands at 20, 10, and 3 kb and, with *Pst* I digestion, bands at 15, 6, and 3.8 kb. Because there was only a small number of genomic DNA restriction fragments homologous to pOK6, it appears likely that there is only one or possibly a few genes in mouse that are related to the probe.

DISCUSSION

By using a method capable of identifying rare cDNA clones, it has been possible to clone a cDNA representation of TdT mRNA. The cloned DNA hybridized to a single 2200-nucleotide mRNA in those B- and T-lymphoid cells that contain the enzyme. Those cells tested that lacked TdT also lacked the mRNA. Thus, TdT expression in these cell lines appears to be regulated at the mRNA level, presumably by modulation of transcription. Only a few genomic DNA restriction fragments hybridized to the TdT cDNA clone, suggesting that TdT is not a member of a multi-gene family. The size of mRNA found in various cells and the small amount of TdT-related DNA suggests that the TdT in B- and T-lymphoid cells could be encoded by the same gene. Therefore, TdT would represent a gene selectively expressed in both immature B- and T-lymphoid cells but not in mature cells, making it interesting to further study its regulation. The selective expression of the TdT gene early in B- and T-lymphoid cell differentiation is consistent with its postulated role of inserting the *N* region into DNA joins.

The frequency of TdT mRNA molecules can be estimated from the percentage of positive clones detected by antibody screening of the λgt11 cDNA library (0.0025%), but it is first necessary to consider the efficiency of the antibody screening procedure. When inserted into the *Eco*RI site of λgt11, one out of six cDNA molecules should be in the appropriate phase and orientation to allow translation of β-galactosidase. Assuming that antibody screening is perfectly efficient, 6 × 0.0025% or 0.015% of the λgt11 phage in the cDNA library should contain inserts. In fact, rescreening the library with a radioactive pTdT20 probe showed that 0.01% of the clones were positive (data not shown). Thus, the antibody screening procedure detected most in-phase TdT cDNA-containing clones. These data show that about 10^{-4} of the mRNA molecules in RL♂11 cells encode TdT, and it is evident that the antibody screening could easily detect a mRNA of this abundance or even less. This sensitivity underscores the power of the λgt11 system when used with a polyclonal antibody.

The antibody probe recognized the β-galactosidase-cDNA fusion protein in the phage plaques of each positive clone. It was surprising then that the fusion protein synthesized by the phage whose cDNA insert was used most extensively in these studies, phage 20, showed no reactivity with antibody after electrophoretic fractionation and transfer to nitrocellu-

lose. However, the β-galactosidase-cDNA fusion proteins synthesized by phage 3 and phage 12, whose nucleotide sequences overlapped with that of phage 20, bound the antibody. This discrepancy may indicate a general precaution that should be taken when comparing results obtained by antibody screening of phage plaques with those obtained by blotting of protein that has been fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis. In the former case, protein is bound to nitrocellulose without prior denaturation; in the latter case, proteins are denatured by boiling in $NaDodSO_4$ and 2-mercaptoethanol prior to electrophoresis and transfer to nitrocellulose. In some cases, protein bound to nitrocellulose after such processing may not present antigenic determinants available for antibody binding, possibly because of improper renaturation of the protein after transfer to the filter.

Although it is not unambiguously proven here that the protein translated in the hybrid selection experiment was TdT and not a protein against which there is contaminating activity in the antiserum, there are several arguments strongly favoring its identity as TdT. First, the antibody was prepared against the low molecular weight form of TdT, which would not likely be contaminated with significant amounts of a M_r 58,000 protein. Second, binding of purified low molecular weight TdT to the antibody before immunoprecipitation abolished the ability of the antibody to immunoprecipitate the M_r 58,000 band (unpublished observations; ref. 18). Thus, those antibodies that bound purified TdT also bound the M_r 58,000 band. Third, the mRNA encoding the postulated contaminant would have to show a cell type-specific expression identical to that of TdT. In the case of cell lines 40E4 and 22D6, such specificity would be particularly unlikely, given the relatedness of the two cell lines. In fact, it is not at all clear why two cell lines of such similar properties should differ so markedly in their TdT content.

The TdT cDNA should be used to facilitate understanding of the expression and structure of the gene for TdT. In addition, introduction of a functional TdT gene into cells should provide insights into the role of TdT in lymphocyte maturation.

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