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Cloning and comparative analysis of the human pre-T-cell receptor α-chain gene

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ABSTRACT **In immature T cells the T-cell receptor (TCR) β-chain gene is rearranged and expressed before the TCR α-chain gene. At this stage TCR β chain can form disulfide-linked heterodimers with the pre-T-cell receptor α chain (pTα). Using the recently isolated murine pTα cDNA as a probe, we have isolated the human pTα cDNA. The complete nucleotide sequence predicts a mature protein of 282 aa consisting of an extracellular immunoglobulin-like domain, a connecting peptide, a transmembrane region, and a long cytoplasmic tail. Amino acid sequence comparison of human pTα with the mouse pTα molecule reveals high sequence homology in the extracellular as well as the transmembrane region. In contrast, the cytoplasmic region differs in amino acid composition and in length from the murine homologue. The human pTα gene is expressed in immature but not mature T cells and is located at the p21.2–p12 region of the short arm of chromosome 6.**

Intrathymic T-cell development is accompanied by ordered rearrangement of T-cell receptor (TCR) genes as well as by changes in expression of surface markers (1). At a certain developmental stage, $CD4^-$, $CD8^-$, $CD3^{low}$, $CD44^+$, $CD25^+$ T-cell precursors downregulate CD44 expression and begin to rearrange the TCR β-chain (TCRβ) locus. After productive TCRβ genes are formed, thymocytes become $CD25^-$, acquire CD4/CD8 coreceptors on the cell surface, and rearrange the TCRα locus (2–5). At the $CD4^+$ $CD8^+$ $TCR\alpha\beta^+$ stage, thymocytes are subjected to positive and negative selection depending on the quality of TCR–major histocompatibility complex ligand interaction (6, 7). While the TCRαβ controls late thymic development, early developmental steps are controlled by the pre-TCR composed of the variant TCRβ chain that is disulfide linked to the invariant pre-TCRα (pTα) protein recently described in mice (7–9). The TCRβ–pTα heterodimer is associated with CD3 molecules (10) and signals triggered by the pre-TCR induce expansion and differentiation of immature precursor cells (8, 9, 11). Here we report on the isolation and characterization of the human pTα cDNA and its comparison with the murine homologue.¶ In addition we provide data on the chromosomal location as well as on pTα expression in thymic subsets.

MATERIALS AND METHODS

cDNA Isolation and Sequencing. A 3-year-old human thymus cDNA library constructed in λgt10 vector (1×10^6 clones) (Clontech; HL1127A) was screened with a probe corresponding to the full-length murine pTα. Prehybridization and hybridization were done at 65°C in a solution containing 1 M NaCl, 50 mM Tris·HCl (pH 7.2), 10% dextran sulfate, 1% SDS, and 0.250 mg of salmon sperm DNA per ml. Filters were washed in 2× SSC first at room temperature and then at 65°C. The murine cDNA was used as a probe and labeled with [α-^{32}P]dCTP by random priming (DNA labeling kit; Boehringer Mannheim). Inserts from positive clones were subcloned in the *Eco*RI site of pBluescript SK(−) vector. Plasmid DNA was purified according to Birnboim and Doly (12) for double strand and according to Sambrook (13) for single strand. Dideoxynucleotide sequencing was carried out either on single-stranded template or on double-stranded template obtained from the pBluescript subclone. Reading ambiguities were resolved by substitution of 7-deaza-dGTP and 7-deaza-dATP for dGTP and dATP, respectively, in sequencing reaction mixtures. Sequenase from United States Biochemical and T7 DNA polymerase from Pharmacia Biotech have been used.

DNA sequence analysis was done with the GCG program (Genetics Computer Group, program manual for the GCG package, version 7, April 1991). Homology searches in the EMBL (release 42.0, March 1995), GenBank (release 88.0, April 1995), and Swiss-Prot (release 29.0, June 1994) data banks were done with the FASTA (14) program. Prediction of the secondary structure and hydrophilicity searches were done with the PEPTIDESTRUCTURE program.

Cloning of the 5′ and 3′ Ends. $Poly(A)^+$ RNAs were extracted from 3-month-old human thymus by using the citric acid method (15). cDNA synthesis was performed on 1 μg of $poly(A)^+$ RNA with an oligo(dT) adaptor 5′-GACTCGAG-$TCGACATCGAT_{17}$-3′ according to the supplier's recommendations (cDNA cycle kit; Invitrogen). To obtain the 3′ end, a primer specific for the human pTα sequence (5′-CCTGCCT-TCTGAGGAGCTG-3′) as deduced from analysis of the positive λgt10 recombinant clone and the oligo(dT) adaptor were used for PCR. Thirty cycles of amplification (94°C for 40 sec, 50°C for 2 min, and 72°C for 3 min) were carried out followed by a 10-min final extension at 72°C in 100 μl of PCR mixture containing 2 μl of cDNA and 50 pmol of each primer. A second round of amplification was performed on amplified material after size purification. The primers used for the second amplification were a nested pTα-specific primer (5′-TGGGGCTGAG-GGTCACAGCA-3′) and the adaptor (5′-GACTCGAGTCGA-CATCGATT-3′). The amplified material was migrated on a 1% agarose gel, purified by adsorption on glass beads, and ligated into the pCR-Script SK vector (Stratagene).

For the 5′ end amplification, the cDNA was synthesized using the pTα-specific primer 5′-GCCTCTCCTGACAGAT-GCAT-3′. The dG-tailing was performed in a final vol of 50 μl of tailing buffer with 15 units of terminal deoxynucleotidyltransferase (GIBCO/BRL). The first amplification of dG-

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Abbreviations: TCR, T-cell receptor; TCRβ and -α, TCR β chain and TCR α chain; pTα, pre-TCRα; PKC, protein kinase C; C, constant; V, variable.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U36759).

tailed cDNA was performed in 100 μl of PCR buffer using as primers an anchor poly(C) primer (16) and a human pTα-specific primer (5′-GGGAGAGATGGGCCAAGTTG-3′). Amplification was done for 25 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2.5 min. A second round of amplification was performed on size-selected amplified material with a nested pTα-specific primer (5′-TGATTGGTGGGGCCAGAGAAG-3′) and the adaptor primer (16). The PCR product was cloned as described above.

Southern Blot. A Zoo-Blot from Clontech was hybridized either with the entire human pTα cDNA, with a 300-bp *Pst* I insert coding for the human pTα cytoplasmic tail, or with a PCR product corresponding to the murine cytoplasmic sequence. Prehybridization and hybridization were done at 65°C as described. Membranes were washed in 2× SSC/0.1% SDS at room temperature and then at 65°C. Stringent washes were performed in 0.5× SSC/0.1% SDS at 65°C when the Zoo-Blot was hybridized with the entire probe. Probes were removed in 0.5% SDS at 90–100°C.

Monoclonal Antibodies. The following monoclonal antibodies were used in this study: phycoerythrin-coupled Leu-3a (anti-CD4 antibody; Becton Dickinson), fluorescein isothiocyanate (FITC)-labeled Leu-4 (anti-CD3 antibody; Becton Dickinson), biotinylated Leu-2a (anti-CD8 antibody; Becton Dickinson).

Surface Staining and Cell Sorting. Cells were suspended in PBS containing 2% fetal calf serum (FCS) at 10^7 cells per ml and incubated with the desired antibodies at optimal concentration for 10–20 min on ice. After incubation, cells were washed twice in PBS plus 2% FCS and resuspended either in the same solution (in the case of direct staining with phycoerythrin-conjugated, FITC-conjugated monoclonal antibodies) or (in the case of indirect staining with biotinylated antibodies) in optimally diluted APC (Southern Biotechnology Associates) and incubated for an additional 10–20 min on ice. Cell suspensions were filtered and sorted on FACStar^{PLUS} (Becton Dickinson) instruments. Sorted populations were reanalyzed to test their purity, which was always found to be >99%.

Reverse Transcription PCR. Cells (5×10^4) were directly sorted into 500 μl of RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX); nonsorted cells were washed in PBS and resuspended in RNAzol. Total RNA was extracted according to the manufacturer's protocol. cDNA was prepared with random hexamer primers and reverse transcribed with a Superscript kit (GIBCO/BRL). For PCR, primers used were oligonucleotides recognizing sequences in the 5′ and 3′ regions of the pTα (5′-GGCACACCCTTTCCTTCTCTG-3′ and 5′-GCAGGTCCTGGCTGTAGAAGC-3′) and actin (5′-ACACTGTGCCCATCTACGAGGGG-3′ and 5′-ATCATGGAGTTGAAGGTAGT TTCGTGGAT-3′) cDNAs. PCRs were done in 30 μl of reaction mixture containing 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 10 mM mixed dNTP, 10 pM each oligonucleotide primer, and 1 unit of AmpliTaq DNA polymerase (Roche Diagnostics). DNA was amplified for 35 cycles at an annealing temperature of 55°C with a thermal cycling machine (Perkin–Elmer/Cetus). A 9-μl portion of each amplified product was separated throughout a 1% agarose gel by electrophoresis and stained with ethidium bromide.

Chromosomal Localization by *in Situ* Hybridization. *In situ* hybridization was carried out on chromosome spread preparations obtained from phytohemagglutinin-stimulated human lymphocytes cultured for 72 hr. 5-Bromodeoxyuridine was added for the final 7 hr of culture (60 μg per ml of medium) to ensure a posthybridization chromosomal banding of good quality. The pTα clone containing an insert of 800 bp in pBluescript was ^{3}H labeled by nick-translation to a specific activity of 1.4×10^8 dpm·μg^{-1}. The radiolabeled probe was hybridized to metaphase spreads at a final concentration of 25 ng per ml of hybridization solution as described (17).

After coating with nuclear track emulsion (Kodak NTB_2), the slides were exposed for 21 days at +4°C and then developed. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered Giemsa solution and metaphase spreads were photographed. R-banding was then performed by the fluorochrome/phytolysis/Giemsa method and metaphase spreads were rephotographed before analysis.

RESULTS

Isolation and Analysis of Human pTα cDNA. Screening of a human thymus cDNA library led to the isolation of three positive clones, which were subcloned into pBluescript SK(−) and sequenced. Sequence analysis of a 931-bp insert from the longest clone (F6) revealed the presence of a long open reading frame. To obtain the complete sequence of the human pTα and to confirm it independently, a rapid amplification of cDNA ends (RACE) (18) was undertaken with primers located in the immunoglobulin-like domain. Sequencing of 3′ RACE products allowed the identification of 104 additional nt with one putative polyadenylylation signal 20 bp upstream of the poly(A) tail. Sequencing of the 5′ products identified 60 additional nt in the 5′ untranslated region. By the combination of these techniques a sequence of 1097 nt was obtained. These data are in agreement with the 1.1-kb band observed in Northern blots of human thymus mRNA hybridized with mouse or human pTα probe (data not shown). The included open reading frame contained 846 nt encoding a 282-aa protein including the N-terminal methionine (Fig. 1). The calculated molecular weight of the protein was 27,793. The overall identity with the murine pTα was 63%. Positionwise, the human ATG that heads a long open reading frame corresponds to the second in-frame ATG in the murine pTα sequence, suggesting that in the mouse the second ATG in the 5′ region represents the initiator codon.

The deduced human amino acid sequence reveals a 5′ hydrophobic region of 16 aa with 75% identity at the nucleotide level and 70% identity at the amino acid level with the murine pTα leader sequence. The following 130 aa show 83% identity with three cysteines at the conserved positions 31, 91, and 119. The putative extracellular region presents features of an immunoglobulin-like structural constant (C) domain as defined by Williams and Barclay (19). The sequence encompassing aa 1–116 shows the characteristic seven β-strands, which could fold in two β-sheets stabilized by an intrachain disulfide bond formed by the cysteines at positions 31 and 91 and a tryptophan in position 46 that is involved in establishing the tertiary structure (20). This structural organization is very well conserved between mouse and human. While the murine extracellular domain exhibits two potential sites of glycosylation, the human sequence contains only one. The conserved cysteine at position 119 is assumed to participate in formation of the interchain disulfide bond with TCRβ and is included in a small sequence of 15 aa that form the connecting peptide, which shows only 60% identity to the murine sequence. The following 20 aa share 80% identity to the murine transmembrane region. They are mostly hydrophobic and the region contains two polar residues (arginine and lysine) that are separated by the same 4 aa observed in the murine sequence. This feature is also conserved in human and murine TCRα chains and is assumed to be essential for the assembly and transport of the TCRαβ–CD3 complex (21).

The homology between human and murine pTα ends abruptly 5 aa after the predicted transmembrane region. Furthermore, the length of the human pTα cytoplasmic tail (114 aa) differs from that of the mouse (31 aa). There are three potential protein kinase C (PKC) sites in this part of the human pTα as opposed to two in the mouse, which correspond positionwise to the two first PKC sites in the human pTα.

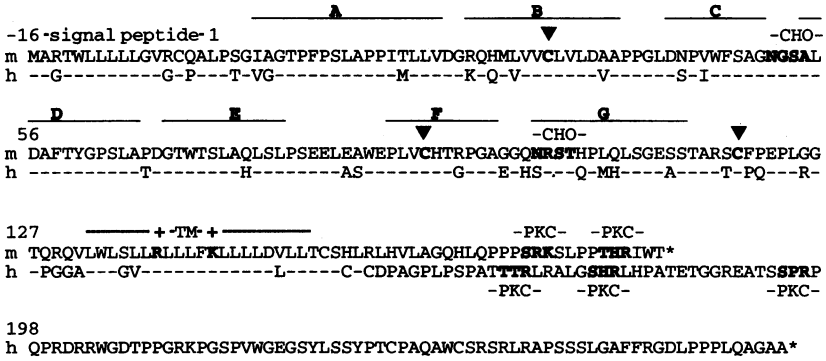

FIG. 1. Comparison of the deduced amino acid sequence encoding the human and mouse pTα cDNA. Human sequence (h) is aligned with the previously described murine pTα sequence (m) (accession no. U16958). Amino acid residue numbers start with −16 as the first position of the leader and proceed from +1 of the putative mature proteins; locations of the potential N-linked glycosylation site (CHO), the predicted leader (peptide signal), the transmembrane (TM), and the potential PKC phosphorylation sites are indicated. The three cysteines at positions 31, 91, and 119 are marked (▼). The conserved basic amino acids arginine and lysine, located in the transmembrane region, are indicated (+) in the lower line. The conserved position of the β-strands (A, B, C, D, E, F, and G) deduced by computer analysis of the secondary structure of the murine and the human sequence are shown above the two sequences. Hyphens replace residues in the human pTα that are identical to the corresponding residues in mouse pTα.

Unlike the murine pTα, the human sequence does not contain a PPGHR CD2-like motif, which is believed to play a role in CD2-mediated T-cell activation (22). Except for a proline triplet at the 3′ end, the human pTα cytoplasmic tail does not contain a proline-rich region that could constitute an SH3 domain binding region (23), as found in the murine pTα.

Interspecies pTα Hybridization. To determine whether nucleotide sequence homologies could be detected by cross-hybridization at low stringency, we performed Southern blot analysis of *Eco*RI-digested DNA from several species. When the entire human pTα cDNA was used as a probe at least one hybridizing band was detected in all species analyzed. The same result was obtained with a probe corresponding to the human extracellular immunoglobulin-like domain only. In contrast, with probes corresponding to the cytoplasmic tails of either human or murine pTα, hybridization was obtained only with human and monkey DNAs or murine and rat DNAs, respectively (Fig. 2). Thus, among different species, the extracellular immunoglobulin-like domain appears much better conserved than the cytoplasmic tail, a result consistent with the sequence data of human and murine pTα.

Human pTα Chromosomal Location. In 100 metaphase cells examined after *in situ* hybridization, there were 214 silver grains associated with chromosomes and 58 of these (27.1%) were located on chromosome 6; their distribution on chromosome 6 was not random: 44 of the 58 silver grains (76%) were localized on the p21.2–p12 region of the short arm of chromosome 6 (Fig. 3). Thus, the human pTα gene maps to the 6p21.2–6p12 region of the human genome.

Recently, the murine pTα gene was localized in the D.E1 region of chromosome 17 (24), which is homologous to the short arm of human chromosome 6. Thus, in both human and mouse the pTα gene is localized in the area of the major histocompatibility complex.

Intrathymic Expression of Human pTα. Human pTα RNA was detected by PCR analysis using the primers described. Human thymocytes were separated into immature $CD4^-$ $CD8^-$, $CD3^{low}$, $CD4^+$ $CD8^-$, $CD3^{low}$, $CD4^+$ $CD8^+$, $CD3^+$ as well as mature $CD4^+$ $CD8^-$ $CD3^{++}$, $CD4^-$ $CD8^+$ $CD3^{++}$, and $CD4^-$ $CD8^-$ $CD3^{++}$ thymocytes (25, 33). As shown in Fig. 4, pTα RNA is detected in the immature but not the mature subsets, and its expression is strongest in the $CD4^+$ $CD8^-$ $CD3^{low}$ fraction of cells that are on their way to differentiate into $CD4^+$ $CD8^+$ thymocytes.

DISCUSSION

In this paper, we report the nucleotide sequence of the human pTα cDNA. Analysis of the human and murine pTα cDNA shows the existence of conserved and nonconserved regions in the protein that may be related to its function. Alignment of the extracellular regions of the human and mouse pTα shows that they are identical in length. The highest degree of homology is detected in the peptidic stretch corresponding to the immunoglobulin-like C domain (20), where 83% of identity

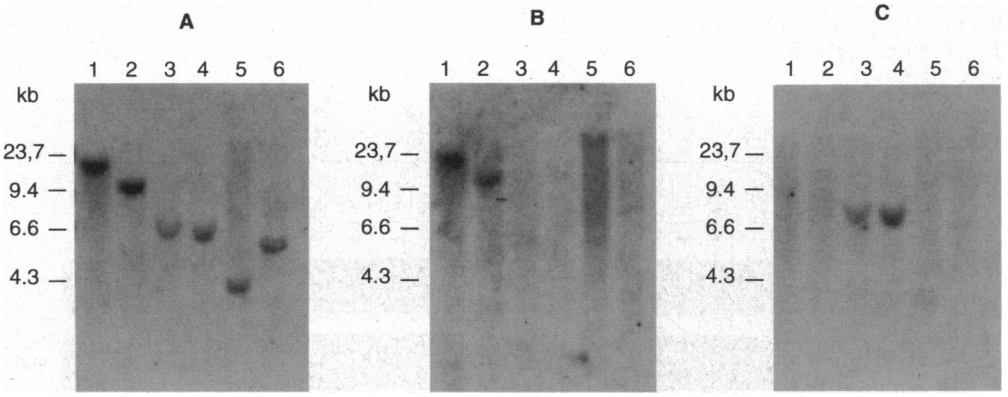

FIG. 2. Interspecies Southern blot analysis. *Eco*RI-digested DNA from human (lanes 1), monkey (lanes 2), rat (lanes 3), mouse (lanes 4), dog (lanes 5), and cow (lanes 6) was analyzed by Southern blotting. The blot was successively hybridized with probes corresponding to the entire human pTα cDNA (*A*) and part of the cDNA encoding the cytoplasmic tails of human (*B*) and mouse (*C*) pTα.

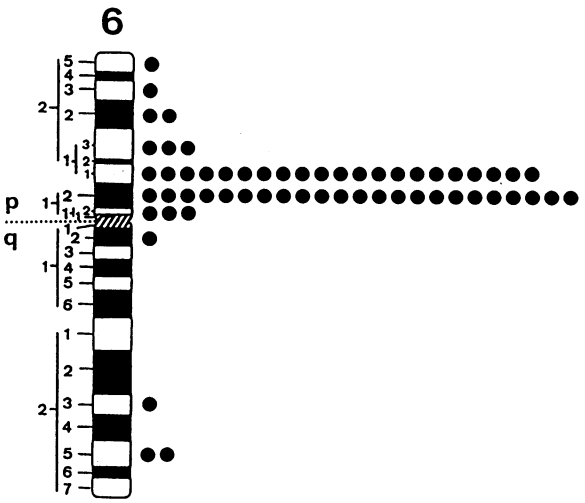

FIG. 3. Idiogram of the human G-banded chromosome 6 illustrating distribution of the labeled sites with the human pTα probe.

is observed. This score is greater than the identity found between the human and murine C regions of TCRα (≈60%). This remarkable conservation in the extracellular region of pTα could reflect the constraints imposed by the interaction with a conserved thymic (or extrathymic) ligand, which could promote expansion and differentiation of the T-cell lineage through signal transduction by the pre-TCR. Alternatively, the conservation could be due to pairing with the TCRβ protein. Some of the conservation could also be imposed because, as in pre-B cells, an additional variable (V) domain in pre-T cells might be noncovalently associated with the immunoglobulin-like domain of pTα. This could be compatible with the notion that the TCRβ–pTα heterodimer is inefficiently transported to the cell surface in a mature T-cell line (9). On the other hand, there are several pieces of evidence that suggest that the structure of TCRβ differs from that of the immunoglobulin heavy chain. V_β and C_β are not structurally autonomous units (26) unlike the V and the C regions of immunoglobulin heavy chain (27). This is confirmed by analysis of the three-dimensional structure of TCRβ (28), showing that the V_β and C_β domains are in intimate contact in the crystal structure. The V_β is folded on the C_β domain in a rigid conformation, indicating that the interaction between the V regions of the α and β chains could be reduced. Because of the stability of TCRβ by itself, there may be no requirement for an associated V pre-T-cell region in the pre-TCR. Furthermore, it has been shown that expression of a mutilated V_β gene permits the intrathymic double-negative to double-positive transition to occur (29). This could suggest that the V domain of TCRβ is not an essential part of the pre-TCR complex to achieve signal transduction and, therefore, that interaction of a V pTα domain with the V_β domain, analogous to the binding of V pre-B cell to V_μ in pre-B cells, is not needed to promote differentiation. These considerations are at least consistent with the idea that the pTα–TCRβ complex could be sufficient to drive thymocyte development without the association of a V-like domain.

The peptide connecting the pTα transmembrane region with the immunoglobulin-like domain shows less homology (60%) but contains a conserved cysteine in position 119, expected to participate in the disulfide bond with TCRβ. A high degree of conservation is observed in the transmembrane region of pTα, which contains the basic amino acids arginine and lysine that are also found in TCRα and TCRδ and appear necessary for interaction with the CD3 molecules (21). These data indicate that the human pTα molecule shares with its mouse counterpart all the features necessary to form a complex with TCRβ and the CD3 complex. It has been reported (10) that a weak association exists between the pre-TCR αβ and the ζ chains of the CD3 complex. A highly conserved phenylalanine at position 195 of the TCRα C region has been shown to be essential for a high avidity interaction with the ζ chains (30, 31). The absence of this phenylalanine residue in the corresponding part of the extracellular regions of human and murine pTα could account for the poor association of pre-TCR αβ chain with the ζ chain.

The homology between human and mouse pTα breaks off abruptly at the end of the transmembrane region. In fact, the 127 aa that are predicted to compose the human cytoplasmic region do not show any significant identity with the 30 aa residues found in the mouse pTα cytoplasmic tail. In this region of the human molecule, in addition to the presence of potential PKC sites present in the two molecules, we could identify neither a CD2-like motif (PPGHR) (22) nor proline-rich regions potentially involved in SH3 binding (23).

These results argue against the previously discussed hypothesis (9) that the cytoplasmic part of pTα could play a role in signal transduction of the pre-TCR through binding with proteins bearing SH3 domains like $p56^{lck}$. Indeed, the fact that pre-TCR is associated with the signal transduction CD3 molecules and that the phosphorylation pattern of T-cell activation is very similar after pre-TCR or TCRαβ crosslinking (unpublished observations) could suggest that the cytoplasmic pTα tail has no special role in signal transduction.

The lack of identity between the human and murine cytoplasmic tails could reflect a divergence in gene structure. In contrast to the human sequence, the murine pTα sequence contains a B2 repetitive element in the 3′ untranslated region that provides the polyadenylylation site. This type of element is known to generate recombination or translocation events by insertional mutagenesis. Moreover, a difference between the gene structure of the murine pTα gene and the TCRα and TCRδ genes is that the pTα transmembrane region, the cytoplasmic tail, and the 3′ untranslated region are encoded by a single exon (24), whereas in TCRα and TCRδ genes the 3′

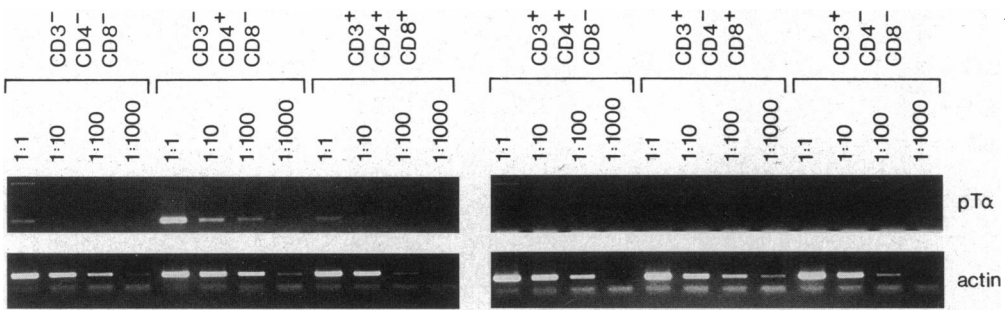

FIG. 4. Semiquantitative analysis of pTα expression on human thymocyte subsets. PCR amplifications of pTα- and actin-specific sequences were performed on serial dilutions of cDNAs. Amplified material was separated by agarose gel electrophoresis and stained with ethidium bromide. Phenotypes of the thymic populations are indicated.

untranslated region is encoded by a separate exon. These differences could suggest that the 3′ part of the pTα molecule has been subjected to variation. Analysis of the genomic organization of the human pTα should help to define whether differential splicing sites exist between human and murine pTα.

The Zoo-Blot analysis indicates again that a portion of the extracellular part of pTα is conserved in all the species analyzed and is compatible with the idea that some type of selective pressure may have conserved the extracellular domain of the pTα but not the cytoplasmic region.

Overall, the human and murine pTα sequences for both the extracellular and cytoplasmic regions show no similarities with any published sequences in the data banks. We did not find any evidence for the existence of a pTα pseudogene when screening the library or in Southern blot analysis. Thus, unlike the λ5 gene (32), the pTα gene may not belong to a cluster of similar genes.

The chromosomal location of pTα on the short arm of human chromosome 6 was expected after it was shown that the murine pTα gene is located in the D/E1 region of chromosome 17. In fact, these regions are syntenic and harbor the major histocompatibility complex locus.

Finally, the expression pattern of pTα in the human thymus corresponds to that previously observed in the murine thymus. Taking into consideration the differences in thymocyte surface markers in the two species, our data indicate that in both human and mouse pTα expression is developmentally regulated.

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