Evolution of Transglutaminase Genes: Identification of a Transglutaminase Gene Cluster on Human Chromosome 15q15

STRUCTURE OF THE GENE ENCODING TRANSGLUTAMINASE X AND A NOVEL GENE FAMILY MEMBER, TRANSGLUTAMINASE Z*

Received for publication, March 21, 2001, and in revised form, June 1, 2001

Published, JBC Papers in Press, June 4, 2001, DOI 10.1074/jbc.M102553200

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We isolated and characterized the gene encoding human transglutaminase (TG)X (TGM5) and mapped it to the 15q15.2 region of chromosome 15 by fluorescence in situ hybridization. The gene consists of 13 exons separated by 12 introns and spans about 35 kilobases. Further sequence analysis and mapping showed that this locus contains three transglutaminase genes arranged in tandem: EPP42 (band 4.2 protein), TGM5, and a novel gene (TGM7). A full-length cDNA for the novel transglutaminase (TG) was obtained by anchored polymerase chain reaction. The deduced amino acid sequence encoded a protein with 710 amino acids and a molecular mass of 80 kDa. Northern blotting showed that the three genes are differentially expressed in human tissues. Band 4.2 protein expression was associated with hematopoiesis, whereas TGX and TGR showed widespread expression in different tissues. Interestingly, the chromosomal segment containing the human TGM5, TGM7, and EPP42 genes and the segment containing the genes encoding TGC, TGE, and another novel gene (TGM6) on chromosome 20q11 are in mouse all found on distal chromosome 2 as determined by radiation hybrid mapping. This finding suggests that in evolution these six genes arose from local duplication of a single gene and subsequent redistribution to two distinct chromosomes in the human genome.

* This work was supported by 1996 Swiss National Science Foundation Fellowship 823A-046620 for advanced researchers (to D. A.) and Arthritis Research Campaign of the UK Grant A0560. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF206502-AF206512 (genomic sequences) and AF363393 (cDNA sequence).

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‡ The abbreviations used are: TG, transglutaminase (see Table I); kb, kilobase pair(s); BAC, bacterial artificial chromosome; PCR, polymerase chain reaction; bp, base pair(s); RACE, rapid amplification of cDNA ends; nt, nucleotide(s); LOD, log of the ratio of odds.
a-subunit and TGK (12). Considering the similarity in gene structure, protein primary structure, and three-dimensional folding (20–22) as well as catalytic mechanism (23), transglutaminase genes seem derived from a common ancestral gene, which itself is related to cysteine proteases (24). Transglutaminase genes were thought to be scattered in the human genome, because the genes encoding TGK and factor XIII a-subunit have been mapped to chromosome 3 (29, 30).

We recently isolated a cDNA encoding a novel member of the transglutaminase gene family, TGX, from human foreskin keratinocytes (4). Two related transcripts with apparent sizes of 2.2 and 2.8 kb were obtained that encoded proteins of 638 and 720 amino acids with molecular masses of 72 and 81 kDa, respectively. We now have characterized the structure of the gene encoding TGX and shown that the two previously isolated gene products result from alternative splicing of exon 15. Three of the cDNA have been cloned to 15q15.2 region of chromosome 15. Analysis of its flanking sequences revealed the presence of a cluster of three transglutaminase genes within ~100 kb, including the gene encoding band 4.2 protein and a novel transglutaminase gene (TG7). We have further characterized the new transglutaminase, TG7, encoded by the TG7 gene by determining its primary structure and tissue distribution. Our findings provide new insights into the evolution of the transglutaminase gene family.

**MATERIALS AND METHODS**

**Reagents**—Oligonucleotides were from Oligo Etc., Inc. (Wilsonville, OR) or Life Technologies, Inc., and restriction enzymes were from Promega (Madison, WI). Image clones with GenBank accession numbers AI018564, AI024635, and AW511368 were obtained from Genome Systems, Inc. (St. Louis, MO). Reagents for cell culture were from Life Technologies, Inc.

**Genomic Library Screening**—A human BAC library established in an F-factor-based vector, pBeloBAC 11, and maintained in Escherichia coli DH10B (31) was screened by PCR (Genome Systems). A 147-bp DNA fragment unique to TG7 (4) was amplified from 100 ng of genomic DNA in a total of 100 μl of reaction buffer containing 2 μM MgCl2, 0.2 mM dNTPs, and 50 pmol of the desired oligonucleotide primers. PCR cycles (Robocycler) were 45 s at 94°C (denaturation), 1 min at 60°C (annealing), and 5 min at 72°C (elongation) for a total of 32 cycles. The following oligonucleotides were used as upstream and downstream primers, respectively, in the individual reactions: intron 2: 5′-GGACCACTCCTGTTGTCGGG and 5′-GGGCGGCTGGGCTGTGATGGCGTG; intron 3, 5′-ACCTCTTGAA-AATCCACATCAGCTCT and 5′-CAGTCTCTCGCTGCTCTTATGGAAGC; intron 4, 5′-GACAGTGAAACCACAGGCGAG and 5′-TGTTGACTGTCCTGAGTTC; intron 5, 5′-GCGTCCAGCACCTGCCAGCATCC (forward) and 5′-GGTTTGCTGCCCTCTTGCAAATGCCT (reverse) and 5′-AGGGGGAATGAGACACTTGGAC (reverse). Primers were synthesized by using the GlassMax DNA isolation kit (Life Technologies, Inc.) and deoxynucleotidyl transferase (Promega) for 30 min at 37°C.

**Rapid Amplification of 5′-end PCR** protocols (4) were used to determine the transcription start site and obtain additional sequence information of exon 1 of TG7. Briefly, double-stranded cDNA was prepared from poly(A)+ RNA of cultured normal human keratinocytes (prepared as described previously (4)) with the Copy kit (Invitrogen, San Diego, CA). The cDNA was purified from nucleotides using QIAquick PCR purification columns (QIA, Inc.). It was then subjected to the process of reverse transcriptase (RT) and dilution, and the aliquoted cDNA was subjected to RT-PCR with the abridged primer TG7 and a primers that were specific to TG7 (32). After the transfer of 25% of this reaction at 94°C to a new tube containing abridged anchor primer and TG7-specific primer (see above), the

**Amplification of TG75 Intron Sequences**—PCRs were carried out with 2.5 units of Taq DNA polymerase (Fisher) and 100–200 ng of plasmid DNA from BAC clones in 100 μl of 10 mM Tris/HCl, pH 8.3, 50 mM KCl containing 2 μM MgCl2, 0.2 mM dNTPs, and 50 pmol of the desired oligonucleotide primers. PCR cycles (Robocycler) were 45 s at 94°C (denaturation), 1 min at 60°C (annealing), and 5 min at 72°C (elongation) for a total of 32 cycles. The following oligonucleotides were used as upstream and downstream primers, respectively, in the individual reactions: intron 2: 5′-GGACCACTCCTGTTGTCGGG and 5′-GGGCGGCTGGGCTGTGATGGCGTG; intron 3, 5′-ACCTCTTGAA-AATCCACATCAGCTCT and 5′-CAGTCTCTCGCTGCTCTTATGGAAGC; intron 4, 5′-GACAGTGAAACCACAGGCGAG and 5′-TGTTGACTGTCCTGAGTTC; intron 5, 5′-GCGTCCAGCACCTGCCAGCATCC (forward) and 5′-GGTTTGCTGCCCTCTTGCAAATGCCT (reverse) and 5′-AGGGGGAATGAGACACTTGGAC (reverse). Primers were synthesized by using the GlassMax DNA isolation kit (Life Technologies, Inc.) and deoxynucleotidyl transferase (Promega) for 30 min at 37°C.
first round of amplification was carried out for a total of 37 cycles under the conditions described above except for annealing at 55 °C. Nested PCR was done with the universal amplification primer (Life Technologies, Inc.) and TGX-specific primer P4 (5'-TGAAGTACGGAGGAGTGAAGC-3') as described above (annealing at 60 °C) using 1 μl of the first round PCR.

**Primer Extension Analysis—Oligonucleotide P5, 5'-CATGTAGGCT- GCCTCGGGTCTGCT, containing a 5'-infrared label (IRD 800) was purchased from MWG Biotech (Ebersberg, Germany). Primer P5 (5.3pmol) was hybridized to 1 μl of poly(A)−RNA from primary keratinocytes (4), and reverse transcription was performed with 200 units of Superscript II RNase H−reverse transcriptase (Life Technologies, Inc.) in a total volume of 20 μl for 90 min at 42 °C according to manufacturer instructions. The enzyme was heat-inactivated, and the primer extension products were extracted with phenol chloroform, precipitated with ethanol, and then analyzed on a 4.5% denaturing polyacrylamide gel adjacent to deoxyxynucleotide chain termination sequencing reactions (Thermo Sequenase cycle sequencing kit, Amersham Pharmacia Biotech) derived from a double-stranded genomic DNA fragment using the same primer.

**Southern Blotting—**18 μg of human genomic DNA was digested with BamHI, EcoRI, and HindIII restriction enzymes, separated in a 0.8% agarose gel, and transferred to a Zeta-probe membrane (Bio-Rad). The gel was calibrated using the Lambda DNA/HindIII markers (Promega). 3P-labeled probes were prepared by using a Multiprime DNA labeling system (Amersham Pharmacia Biotech), and PCR products corresponding to intron 2 and exon X were used as DNA templates. Probes were hybridized to the blot overnight at 65 °C in 500 mM NaH2PO4, pH 7.5, containing 1 mM EDTA and 7% SDS. The membrane was washed at 65 °C to a final stringency of 40 mM NaH2PO4, pH 7.5, 1 mM EDTA, and 1% SDS, and the result was developed by exposure of the membrane to BioMax MR film (Eastman Kodak Co.).

**Chromosomal Localization—**Human peripheral blood lymphocytes were used to prepare metaphase chromosome spreads (33). Briefly, cells were cultured in PB-Max karyotyping medium (Life Technologies, Inc.) for 72 h and synchronized by culture in the presence of 10−7 M thymidine (Fluka, Buchs, Switzerland) for another 24 h. Cells were released from the mitotic block by extensive washing and subsequent culture in the above medium containing 10−5 M thymidine for 5 h. The cells were subsequently arrested in metaphase by the addition of colcemid to a final concentration of 0.1 μg/ml (Life Technologies, Inc.). Harvested cells were incubated in 0.075 M KCl for 25 min at 37 °C and fixed in methanol/acetic acid (3:1) solution, and chromosome spreads were prepared by dropping the cells onto the glass slides. After air drying, chromosomes were treated with 100 μg/ml RNase A (10 min) for the activation of the polymerase and the last cycle consisting of band 4.2 sequences was performed as described for the TGX. Long range genomic PCR in combination with direct sequencing from the PAC clones was used to isolate the sequence between the EPB42 and TG5 genes. PCR products were characterized by Pfu Turbo DNA polymerase as described above but using 5% Me2SO and the primers 5'-GATTTACCG-TGTGTCCCATCAGA (forward) and 5'-GTCACCTCTCAACAGGACAAGG (reverse) for 40 cycles in a GeneAmp PCR System 9600 (Perkin Elmer Cetus, Norwalk, CT). A total of 36 PCR cycles were carried out with a combined annealing extension step at 68 °C for 10 min and denaturation at 94 °C for 45 s. The location of the TG5 gene in respect to the TG5–EPB42 gene cluster was confirmed in a similar manner using oligonucleotides 5'-TGGGCGAGGGCCTGAGGTCATCATG (forward) and 5'-GTTTACCTCTCAGGCTTGAGTCCATG (reverse) and the TGX-Plus Long PCR system (Stratagene) according to manufacturer instructions.

**Cloning of TGX by Anchored PCR—**PC-3 cells (European Collection of Cell Cultures, Salisbury, UK) were cultured in the presence of 10−5 M thymidine for 5 h. The cells were harvested by using the Micro-Fast Track 2.0 kit (Invitrogen). cDNA was synthesized from 2 μg of total RNA or 500 ng of poly(A)−RNA by reverse transcription for 1 h at 42 °C using 200 units of Superscript II RNase H−reverse transcriptase and 0.205 μg/μl oligo(dT)12 primer in a 20-μl reaction mixture containing 3.5 μM each of the first strand reaction primers and the second DNA polymerase. The products were purified by using the QIAquick PCR purification kit (Qiagen), and 1 μl of the purified DNA was used as template in the following anchoring PCR. The primer used was 5'-CTTAAAGAACCCGGCCAAAGA-3' (forward) and 5'-GCTGGAGGGCGGGTCTCAGG-3' (reverse) and the TGX-Plus Long PCR system (Stratagene) according to manufacturer instructions. The amplified fragments were used as templates for additional amplification reactions to isolate overlapping DNA fragments, essentially following our previously described strategy (4). Isolated and sequenced TGX fragments in a 5′−3′ direction were obtained with the following primers: 5′-CAACCTTTGGGGCTTGTCTGTG (forward, P8) and 5′-CAGCA-GCTTCAGGGCCTGTTGGT (reverse, P9); 5′-GATCTACCGC- TCTTTGCCCCGTG (reverse, P10), with nested reactions 5′-ATCCATCTGTTGGCAGACCC (forward) and 5′-CAAGATTTAAAAATGAGTAGGAAAGT (reverse) and 5′-CAGCATGTGTTCTACCCCGGCGT (forward, P11) and 5′-GATGTTGTCGATCAGTACAGCAGC (forward, P12), with nested reaction 5′-CTTTAAAGACCCCGCAGAAGAAGCTG (forward) and 5′-TTGTTTTCCTAACCTTCCTGGCTCGG (forward) and 5′-TCTGGACACCTCCTGATACCCAG (reverse); 5′-CTTAGG- GTAATCCACGGCGCAGCAGC (forward, P13) and 5′-GGGGGTACAACACGCAGCAC (forward, P14), with nested reactions 5′-TATTGTTTACGAGGTTCCTC (forward) and 5′-TGCTTGAGTTACAGCTCGTGTGG (reverse). PCR products were performed with 2 μl of the reverse transcription and 1 μg of plasmid DNA, consisting of 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) and the respective primer buffer supplemented with 2 mM MgCl2, 0.2 mM dNTPs, and 25 pmol of each primer in a total volume of 50 μl. 40 PCR cycles were carried out in a GeneAmp 9600 thermal-cycler (Applied Biosystems), each cycle consisting of denaturation at 95 °C for 45 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, with the first cycle containing an extended denaturation period of 2 min. The 5′ end of the cDNA was isolated by 5′-RACE as described above with the exception of using the gene-specific oligonucleotides P9, 5′-TGAAGCTGACCCGGGAGGTAGGAAG (reverse, P15) and 5′-GACAAGTACCAAGCCGCGCTTGTG (forward, P16). Amplified products were analyzed on 1% agarose gels, extracted using the QiAquick gel extraction kit, and cloned as described previously (4). The Northern Hybridization—A human RNA master blot containing poly(A)−mRNA of 50 different tissues was obtained from CLONTECH. The 3P-labeled probes were prepared by random priming labeling of DNA fragments of the different transglutaminase gene products using the Multiprime DNA labeling system. DNA fragments of 500−700 bp comprising the 3′ end of TGX, TGX+, and band 4.2 probes were generated by restriction with PstI and Accl, NdeI and NotI (exons XII and XIII), and XhoI, respectively. The cDNA encoding human band 4.2 protein (34) was provided kindly by Dr. Carl M. Cohen (Boston, MA). Hybridization was performed under the conditions recommended by the manufacturer. The labeled membrane was exposed to BioMax MR film, and the films were developed after 15−24 h for first exposure and 3−5 days for second exposure.

**Amplification of TGX from Different Tissues—**cDNA from various cell lines and human tissue was prepared as described previously (4). A panel of cDNAs from human tissue (multiple tissue cDNA panel I) was obtained from CLONTECH. A 365- or 287-bp fragment of TGX was amplified by PCR using the oligonucleotides 5′-TGGCGAAACCGCCCTGAGGTCATGATAT (forward) and 5′-GTTTACCTCTCAGGCTTGAGTCCATG (reverse) and the TGX-Plus Long PCR system (Stratagene) according to the manufacturer's recommendations.
(reverse), respectively, with an annealing temperature of 60 °C.

Mapping of Transglutaminase Genes in Mouse Genome—The 100 radiation hybrid clones of the 3T1 mouse/hamster radiation hybrid panel (35) (Research Genetics, Huntsville, AL) were screened by PCR. A 139-bp fragment of the tgm5 gene was amplified with primers 5′-TGAGGACTGTTGCTAGGCT (forward) and 5′-TCCGTGCTGTCGGCTAGG (reverse), a 149-bp fragment of the epb42 gene with primers 5′-CAGGGATTATAAAGGAAATTTT (forward) and 5′-TGCAAGGCTCTGACAC (reverse), and a 400-bp fragment of tgm7 with primers 5′-GGAGGGTGGCCCTCATACATT (forward) and 5′-CTTGTAGCCCTCAGTGG (reverse), respectively. PCRs were carried out in a GeneAmp 9600 thermalcycler with 0.035 units/μl AmpliTaq Gold polymerase in standard reaction buffer containing 2 mM MgCl2, 0.2 mM dNTPs, 0.1 μM of each primer, and 2.5 ng/μl genomic DNA in a total reaction volume of 25 μl. Each PCR conditions were: polymerase activation for 10 min at 95 °C, annealing at 60 °C for 45 s, extension at 72 °C for 1 min, and denaturation at 94 °C for 30 s for 35 cycles with a final extension of 3.5 min at 72 °C. PCRs were analyzed by agarose gel electrophoresis using 1 or 1.5% gels. The hybrid cell panel was analyzed at least twice in each case to exclude PCR-related errors. The data were submitted to the Jackson Laboratory radiation hybrid data base for analysis and mapped relative to known genomic markers (www.jax.org/resources/documents/cmdata/rhmap).

RESULTS
Structure of the TGM5 Gene

Isolation of Genomic Clones—A unique insertion of ~30 amino acids between the catalytic core domain and β-barrel domain 1 found in TGX served as a template to design specific primers for screening of a human genomic library. The characterization of several genes of the transglutaminase gene family showed that the positions of the introns have been highly conserved (12, 13, 15, 17–19, 36), and a comparison of the TGX sequence to the sequences of the other transglutaminases indicated that this unique sequence is present within an exon, exon X (see Fig. 7, amino acids 460–503 in TGX). A PCR from human genomic DNA using oligonucleotides P1 and P2, which match sequences at either end of this unique segment, yielded a DNA fragment of the expected size that was confirmed to be the correct product by sequencing (results not shown). Screening of a human genomic DNA BAC library by PCR using these oligonucleotides revealed two positive clones, BAC-33(P5) and BAC-228(P20), which were subsequently shown by Southern blotting with different cDNA probes to contain sequences spanning at least exon II to exon X of the TGM5 gene (results not shown). Restriction analysis further indicated that each of the BAC clones contained substantially more than 50 kb of human genomic DNA.

Gene Structure—The similarity in the gene structure of the different transglutaminase genes prompted us to approach the characterization of introns by PCR amplification using oligonucleotide primers corresponding to the flanking exon sequences at the presumptive exon/intron boundaries. All intron/exon boundaries were sequenced from the PCR products obtained in at least two independent PCRs, where applicable from both BAC clones, to exclude mutations introduced by Taq DNA polymerase, and the results were compared. When sequences of PCR products comprising adjacent introns had no overlap, the intervening sequence (exon sequence) was determined by direct sequencing from isolated BAC plasmid DNA to confirm the absence of additional introns. Similarly, the 3′-untranslated region was obtained by stepwise extension of the known sequence using direct sequencing of BAC plasmid DNA. Both BAC clones terminated short of exon I, and all attempts at isolating clones spanning exon I by the screening of BAC, P1-derived artificial chromosome, and P1 libraries with a cDNA probe or by PCR failed. Exon I and intron 1 sequences were finally derived by nested PCR from human genomic DNA using conditions optimized for long range genomic PCR.

We established that the TGM5 gene is comprised of ~35 kb of genomic DNA and contains 13 exons and 12 introns (Fig. 1). All intron/exon splice sites conformed to the known GT/AG donor/acceptor site rule and essentially to the consensus sequence proposed by Mount (37) (Table II). A sequence homology to the branch point consensus CTGAC (38) was found 44 nt upstream of the 3′ splice site in introns 1, 3–6, and 9–12. The size of the introns varied considerably, ranging from 106 bp to more than 11 kb (Fig. 1, Table III). The sequences obtained from the two different BAC clones matched with the exception of a deletion spanning the sequence from introns 6 to 8 found in BAC-33(P5) (Fig. 1).

Allelic Variants—During the course of this work, we also resequenced the entire coding sequence of TX and found three point mutations as compared with the previously reported cDNA sequence (4). One of the nt exchanges is silent, and the other two result in an amino acid exchange (Table IV). The first two mutations were found in both BAC clones, and the third was present only in BAC-228(P20) because of the deletion in the other BAC clone. These differences might be sequence polymorphisms in the human gene pool, because there was no ambiguity of the cDNA-derived sequence in this position determined from multiple independently amplified PCR products (4). However, the fact that a serine and an alanine residue are changed into a proline and a glycine residue that constitutes the conserved amino acid in these positions in the transglutaminase protein family (see Fig. 7, amino acids 67 and 352 in TGX) suggested that these may have been PCR-related mutations in the cDNA sequence. To clarify this issue, we have prepared cDNA from human foreskin keratinocytes from different individuals, amplified full-length cDNA with high fideli-


**TABLE II**

Splice donor and acceptor sequences in the human TGM5 gene

| Donor sequence | Acceptor sequence |
|----------------|------------------|
| TATA | GGCC |
| AAGT | GCTG |
| GTGAGT | GCAG |
| GCTG | GGGT |
| ATGG | ACTG |

**TABLE III**

Intron sizes and splice types in the human TGM5 gene

| Intron number | Splice type | Size | Method |
|---------------|------------|------|--------|
| 1             | 1          | 6,300 bp | PCR    |
| 2             | 1          | 102 bp   | Sequencing |
| 3             | 1          | 3,300 bp | PCR    |
| 4             | 0          | 2,900 bp | PCR    |
| 5             | 0          | 600 bp   | PCR    |
| 6             | 1          | 11,800 bp | PCR and Restriction Analysis |
| 7             | 2          | 1,600 bp | PCR    |
| 8             | 1          | 106 bp   | Sequencing |
| 9             | 1          | 2,900 bp | PCR    |
| 10            | 1          | 545 bp   | Sequencing |
| 11            | 0          | 1100 bp  | Sequencing |
| 12            | 2          | 209 bp   | Sequencing |

**TABLE IV**

Apparent polymorphisms in the cDNA and genomic DNA sequences for TgX

| Residue | cDNA (Gene) | cDNA* |
|---------|------------|-------|
| 67      | S TCA      | P CCA |
| 220     | Y TAC      | Y TAC |
| 352     | A GCA      | G GGA |

*Additional sequence variant isolated in this work.

...part of the N-terminal β-barrel domain of TgX, and the absence of the sequence encoded by exon 3 is expected to result in major structural changes in at least this domain of the protein. Nevertheless, the expression of TgX in 293 cells using the full-length cDNA resulted in synthesis of two polypeptides with a molecular weight consistent with that of the predicted products from the alternatively spliced transcripts (results not shown).

**5′-Untranslated Region**—Initially, 5′-RACE was used to determine the 5′ end of TgX cDNAs. Transcripts starting at 77, 96, and 157 nt upstream of the initiator ATG were isolated in addition to the previously described shorter transcript (Fig. 2B, arrowheads). All of these transcripts were recovered repeatedly in independent experiments. Finally, primer extension experiments located the major transcription initiation site used in keratinocytes 157 nt upstream of the translation start codon (Fig. 2A). The proximal promoter region was analyzed for potential binding sites of transcription factors using MatInspector (Genomatix, Munich Germany) and GCC (Genetics Computer Group, Inc., Madison, Wisconsin) software packages. No classical TATA-box sequence was found, but a number of other potential transcription factor binding sites could be identified (Fig. 2B), suggesting that the TgX promoter is a TATA-less enhancer. Identification of the start site in keratinocytes was facilitated by the presence of a proximal promoter whose role has been demonstrated in a number of TATA-less genes. c-Myc is found in TATA-less proximal promoters of genes involved in hematopoiesis and often interacts with Ets factors (39), and these sites may be operative in the expression of TgX in hematopoietic cell lines. HEL cells. AP1, Ets, and SP1 elements are typically found in keratinocyte-specific genes (40, 41) and may be involved in transcriptional regulation in keratinocytes. Several AP1 sites are present within 2.5 kb of upstream sequence and could interact with the proximal AP1 factor for activation. SP1 sites are positioned properly upstream of the start points of the shorter transcripts, raising the possibility that these could also be functional in transcription initiation, although to a lesser degree.

**3′-Untranslated Region**—The last exon, exon XIII, contained a consensus polyadenylation signal AATAAA ~600 bp downstream of the termination codon (Fig. 3). This is in good agree-
quences for putative regulatory elements are indicated by asterisk extensions. The termination points of cDNAs isolated from human keratinocytes (4) are underlined. The transcriptional start site is indicated by the arrowhead. The major transcription start site identified by primer extension analysis of poly(A)/H11001 RNA isolated from primary human keratinocytes (4) by primer extension analysis is highlighted with an asterisk.

B

FIG. 2. Structure of the 5′-untranslated region of the human TGM5 gene and mapping of the transcriptional start site. A, primer extension analysis of poly(A) + RNA isolated from primary human keratinocyte prior to lane 1 or after lane 2) culture in suspension. The termination sites of primary keratinocytes mapped by RACE are indicated by arrowheads. The major transcription start site identified by primer extension is highlighted with an asterisk (labeled +1). Consensus sequences for putative regulatory elements are underlined.

ment with the size of the mRNA (2.8 kb) encoding full-length TGX, expressed in human keratinocytes as detected by Northern blotting (4) considering the length of the coding sequence. The TGM5 gene is in close proximity to the EPB42 gene (Table I) has been assigned previously to the 15q15.2 locus (27, 28). This raised the possibility that the TGM5 gene is in close proximity to the EPB42 gene. The TGM5 gene was subsequently localized to chromosome 15 by fluorescent in situ hybridization on human metaphase chromosome spreads using genomic DNA derived from either BAC clone as a probe (Fig. 4A). A comparison of the probe signal to the 4.6-diamino-2-phenyl indole (DAPI) banding pattern localized the TGM5 gene to the 15q15 region. The localization was subsequently refined by determining the distance of the fluorescent signal to the centromere as well as to either end of the chromosome on 13 copies of chromosome 15 and expressing it as a fractional distance of the total length of the chromosome. These measurements placed the TGM5 gene close to the center of the 15q15 region, i.e., to the 15q15.2 locus (Fig. 4B).

**The TGM5 Gene Is Part of a Cluster of Transglutaminase Genes**

Chromosomal Localization of the TGM5 Gene—To address the genomic organization of the TGM5 gene or genes in the human genome, we performed Southern blot analysis of human genomic DNA cut with appropriate size from both, BAC-33(P5) and BAC-228(P20) restriction enzymes using probes derived from intron 2 as well as from the sequence encoded by exon X that is unique to TGX. Bands of 4.3 kb, 4.5, 6.0, and 10.5 kb and 4.5, 9.3, and 2.6 kb were revealed with each probe used in the respective reactions. The simple pattern of restriction fragments hybridizing with the probes indicated that the haploid human genome contains only one TGM5 gene.

**Fig. 3. Structure of the 3′-untranslated region of the human TGM5 gene.** The 3′-flanking sequence is shown with sequences homologous to known consensus sequences for 3′ processing of transcripts (AATAAA, CAYTG, and YGTGGTTYY) underlined. The termination points of cDNAs isolated from human keratinocytes (4) by 3′-RACE are indicated by arrowheads. A pair of inverted long repeat sequences is highlighted in italics.

reported previously that all cDNAs isolated by reverse transcription PCR with an oligo(dT) oligonucleotide from human keratinocytes ended within 9–34 nt downstream of the penultimate nucleotide ATAAA at position 2317 (Fig. 3) (4). It has been shown that this penultimate nucleotide functions as a polyadenylation signal in other genes (42), and it is apparently functional in TGX. Indeed, the most frequently found poly(A) addition sites (indicated in Fig. 3) showed cleavage at a C(A) boundary, which is the preferred sequence for cleavage/polyadenylation in eukaryotic genes (44). However, no CAYTG or YGTGGTTYY signal was found immediately downstream of the ATAAA signal sequence. A possible explanation for these findings is that most transcripts use the ATAAA signal for polyadenylation, as suggested by the Northern blotting data, and occasionally the ATAAA signal functions as a polyadenylation signal and these shorter transcripts are selectively enhanced by PCR amplification because of the smaller size of the PCR product.
Identification of a Novel Transglutaminase Gene, TGM7—We developed a method for the detection and identification of transglutaminase gene products based on reverse transcription PCR of the conserved active site sequence with degenerate primers, and using this method we discovered the gene product of the TGM5 gene (4). Using this same method, we have now identified another new transglutaminase gene product in human prostate carcinoma tissue that we designated TGZ. The full-length cDNA for this new gene product was obtained by anchored PCR using cDNA prepared from the human prostate carcinoma cell line PC-3, essentially following the strategy described previously (4). 5′-RACE was used to determine the 5′ end of the cDNA. The isolated 3′-end sequence was confirmed by sequencing of matching expressed sequence tag clones in the Genbank™ data base (accession numbers AI018564, AI024635, and AW511368). The obtained sequence information consists of

BAC clone that contains all three genes (data not shown). To further confirm the organization of the TGM7 gene in the human genome, we used long range genomic PCR with different combinations of primers designed from the flanking sequences of the TGM5-EPB42 gene and the TGZ cDNA sequence to explore whether the TGM7 gene was present in close proximity to the other two transglutaminase genes. This placed the TGM7 gene −9 kb upstream of the TGM5 gene and demonstrated that the genes are arranged in tandem fashion (Fig. 4C). The sequence between the TGM7 and TGM5 genes was further found to contain a pseudogene of the mitochondrial ATPase D subunit, the relevance of which is unclear.

Determination of the cDNA and Amino Acid Sequence of the TGM7 Gene Product

Based on the initial PCR amplification of a TGZ cDNA fragment from prostate carcinoma tissue, we analyzed prostate-derived cell lines for expression of TGZ by PCR. A full-length cDNA sequence for TGZ was obtained by anchored PCR using cDNA prepared from the human prostate carcinoma cell line PC-3, essentially following the strategy described previously (4). 5′-RACE was used to determine the 5′ end of the cDNA. The isolated 3′-end sequence was confirmed by sequencing of matching expressed sequence tag clones in the Genbank™ data base (accession numbers AI018564, AI024635, and AW511368). The obtained sequence information consists of
The probable initiation codon is present in the sequence GAGATGG, which presents only limited homology to the consensus identified by Kozak (45), which acts as a signal for efficient transcription in eukaryotes. However, the critical purine in position \(3\) as well as the G in position \(4\) are conserved. The signal for polyadenylation (AATAAA) is located \(158\) nt downstream of the termination codon (TGA). The deduced protein consists of \(710\) amino acids and has a calculated molecular mass of \(80,065\) Da and an isoelectric point of \(6.6\).

During the course of the TGz cDNA sequence determination, a number of aberrantly spliced gene products were isolated. These lacked exon VI or part of exon IX (5' end) or retained the whole or part of intron 11. These products are unlikely to be of physiological significance but may point out that the splicing of certain introns in this gene is a difficult and inefficient process. Interestingly, in the variant lacking part of exon IX, splicing occurred from the donor site of exon VIII to an acceptor site in the middle of exon IX, which corresponds to the exact position where an additional intron is present in the \(TGM1\) and \(F13A1\) genes (Fig. 7).

**Evolution of Transglutaminase Genes**

**Mapping of Mouse Genes**—To further analyze the relationship between the most closely homologous genes of the transglutaminase gene family (\(TGM7\), \(TGM5\), and \(EPB42\) on human chromosome 15q15.2 and \(TGM2\) and \(TGM3\) on chromosome 20q11/12), we mapped the respective mouse genes using radiation hybrid mapping. All genes mapped to the distal part of mouse chromosome 2. The genes for \(tgm7\), \(tgm5\), and \(epb42\) showed a best-fit location for the segment defined by D2Mit104.
proximal and D2Mit396 distal, with a highest LOD of 14.5, 16.6, and 15.5 to the anchor marker D2Mit395 (66.9 cM) and an LOD of >20 to D2Ertd616e (69.0 cM). This is in good agreement with the assigned locus of epb42 67.5 cM distal from the centromere (46). The tgm3 gene showed a best-fit location for the segment defined by D2Mit447 proximal and D2Mit258 distal, with a highest LOD of 14.8 and 12.2 to D2Mit258 (78.0 cM) and D2Mit338 (73.9 cM), respectively. The tgm2 gene showed a best-fit location for the segment defined by D2Mit139 proximal (86.0 cM) and D2Mit225 distal (91.0 cM), with a highest LOD of 17.0 to the anchor marker D2Mit287, consistent with its assigned locus 89.0 cM from the centromere (47).

**DISCUSSION**

In this study, we have shown that human TGX is the product of an ~35-kb gene located on chromosome 15q15.2 and containing 13 exons and 12 introns. The intron splice sites conform to the consensus for splice junctions in eukaryotes (37). The transcription initiation site is localized 157 nt upstream of the initiator methionine, and the likely polyadenylation site is localized ~600 nt downstream of the stop codon, which is consistent the previously reported transcript size of about 2.8 kb (4). The short form of TGX, which we have identified in keratinocytes (4), is the product of alternative splicing of exon III. The TGM5 gene is part of a cluster of three transglutaminase genes arranged in tandem, with a novel transglutaminase gene proximal to the TGM5 gene and the gene encoding erythrocyte band 4.2 protein distal. We have used the designation TGM7 for the novel gene and TGZ for its gene product, which is in line with using sequential numbers and letters for genes and proteins, respectively, in the order of discovery.

**Structural and Functional Implications for the Novel Transglutaminase Gene Product Based on Comparative Sequence Analysis**—We have determined the full-length cDNA sequence of the novel transglutaminase gene product, TGZ. A comparison of TGZ to the previously characterized human transglutaminases reveals that the structural requirements for transglutaminase activity and Ca\(^{2+}\) binding are conserved. The structure of several transglutaminases has been solved by x-ray crystallography and shows a high degree of similarity (20–22). The reaction center is formed by the core domain and involves hydrogen bonding of the active site Cys to a His and Asp residue to form a catalytic triad reminiscent of the Cys-His-Asn triad found in the papain family of cysteine proteases (23). The residues comprising the catalytic triad are conserved in TGZ (Cys279, His338, and Asp361) (Figs. 5 and 7), and the core domain shows a high level of conservation as indicated by a sequence identity of ~50% as compared with the other transglutaminases. A Tyr residue in the barrel 1 domain of the a-subunit of factor XIII is hydrogen-bonded to the active site Cys residue, and it has been suggested that the glutamine substrate attacks from the direction of this bond to initiate the reaction based on analogy to cysteine proteases (24). It has further been proposed that this hydrogen bond prevents the formation of a disulfide bond between the active site residue and a neighboring Cys, which would result in enzyme inactivation (22).

**FIG. 6.** Expression of TGX, band 4.2 protein, and TGZ in different fetal and mature human tissues. Human tissue Northern dot blot normalized for average expression of nine different housekeeping genes (CLONTECH) probed with a fragment corresponding to the C-terminal β-barrel domains of TGX (A), TGZ (B), and band 4.2 (C). A diagram showing the type of poly (A)⁺ RNA dotted onto the membrane is shown in D.

**Human TGM5 and TGM7 Genes**

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In TGZ, the Tyr residue has been replaced by a His residue (His538), similar to TGX (Fig. 7). This is expected to be a conservative change, which is supported by our data demonstrating that recombinant TGX produced in 293 cells has transglutaminase activity. The Trp residue (Trp279 in factor XIII), which is thought to stabilize the oxyanion intermediate generated in the proposed reaction mechanism (23), is conserved in TGZ (Trp243). All these residues involved in the catalytic process are conserved in the different transglutaminase gene products with the exception of band 4.2 protein, which is the only member of this gene family without catalytic activity (Fig. 7). Crystallization experiments with factor XIIIa further indicated that four residues are involved in the binding of a Ca\(^{2+}\) ion, including the main chain carbonyl of Ala457 and the side chain carboxyl groups of Asp438, Glu485, and Glu490 (24). All three acidic residues are conserved in TGZ (Asp403, Glu450, and Glu455). Based on the preservation of critical residues for enzyme function and domain folding and the extensive overall similarity of TGZ to the other members of the transglutaminase family with catalytic activity, it is likely that the characterized cDNA encodes an active transglutaminase.

**Organization of Transglutaminase Genes in the Human and Mouse Genome**—Having established the chromosomal localization of the TGM5 and other human transglutaminase genes, it is of interest to compare it with the equivalent chromosomal regions in the genomes of other species and in particular of the mouse, for which many genetic aberrations have been mapped. In this study, we have mapped the gene encoding TGX to chromosome 15q15 by fluorescent in situ hybridization. Band 4.2 protein has been mapped previously to this chromosomal region (27, 28) and subsequently has been assigned to 15q15.2 by expression mapping of the LGMD2A locus on chromosome 15 (48). A short sequence encompassing the left arm of one of the yeast artificial chromosome clones (926G10) used for expression mapping matched with the sequence of intron 12 of the TGM5 gene, which is consistent with our data placing the two genes encoding TGX and band 4.2 in direct apposition. In mouse, the syntenic region is found on chromosome 2, 2F1–F3 (48, 49), and our radiation hybrid data place all three genes, tgm7, tgm5, and epb42, in this segment of chromosome 2. In addition, we have isolated a mouse BAC clone and shown that it contains all three genes. Even though the exact positioning awaits further sequence data, this suggests that all three mouse genes are in close proximity, presumably arranged in a similar fashion to their human counterparts. Our mapping data are consistent with the previously determined location of the epb42 gene by interspecific backcross analysis (46). Data base analysis identified two mutations that are associated with

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2 P. Grenard, and D. Aeschlimann, manuscript in preparation.

3 CEPH-Genethon yeast artificial chromosome 926_g_10 left arm: Fougerousse, F. (GenBankTM/EBI Data Bank accession number X75198).
FIG. 8. Phylogenetic tree of the transglutaminase gene family and genomic organization of the genes in man and mouse. Phylogenetic trees based on the amino acid sequence homology of the gene products have been constructed for the individual domains as well as for the entire gene products using the neighbor-joining method (61) of the PHYLIP or Treecon 1.3b (van de Peer, University of Antwerp, Belgium) software packages. Sequences were aligned using ClustalX and corrected by hand as needed (nonhomologous N- and C-terminal extensions were excluded) to maximize homology as shown in Fig. 7 except including sequences from different species as available. A representative tree based on full-length sequences is shown in A (Treecon, distance estimation according to Tanja and Nej). Bootstrap values are given in percentage and the scale bar reflects 0.05 substitutions per site. In B, a hypothetical pedigree for the gene family is given that is consistent with the data on the sequence relationship of the individual gene products (A) as well as with the data on gene structure and genomic organization (C).

Human TGM5 and TGM7 Genes

| Chromosomal localization | TGM5 (TGα) | TGM7 (TGβ) | EPB42 (band 4.2 protein) | TGM2 (TGβ) | TGM6 (TGα) | TGM3 (TGα) | TGM4 (TGβ) | F13A1 (factor XIII a-subunit) | TGM1 (TGα) |
|--------------------------|------------|------------|-------------------------|------------|------------|------------|------------|----------------------------|------------|
| human                    | 15q15.2    | 15q15.2    | 15q15.2 (17, 26, 4)     | 20q11-12   | 20q11      | 20q11      | 3p21-22    | 6q24-25                   | 14q11.2    |
| mouse                    | 2, 67-69 cM| 2, 67-69 cM| 2, 67-69 cM (46, 4)    | 2, 85-91 cM| 2, 85-91 cM| 2, 85-91 cM| 2, 74-78 cM| 2, 74-78 cM                | 2, 74-78 cM|
| Gene size                | ~33kb      | ~26kb      | ~20kb (13)              | ~27kb      | ~45kb      | ~43kb (14) | ~39kb (14)             | ~169kb (14)         |
| human                    | ~23kb (62) | ~14kb (62) | ~23kb (62)              | ~23kb (62) | ~23kb (62) | ~23kb (62) | ~23kb (62)             | ~23kb (62)         |
| mouse                    |             |            |                         |            |            |            |                         |              |
| Number of exons          | 13          | 13         | 13                      | 13         | 13         | 13         | 15         | 15                        | 15         |
| human                    |             |            |                         |            |            |            |                         |              |
| mouse                    | 13          | 13         |                         |            |            |            |                         |              |

(a) this study
(b) GenBank/EMBL Data Bank with accession number AL031651
(c) GenBank/EMBL Data Bank with accession number AL031678

This locus, ro (rough) and pa (pallid). In fact, it had been suggested that pa might be a mutation in band 4.2 protein (46), but more recently it has been shown that pa/pa mice express normal band 4.2 by cDNA analysis and that the two loci segregate in an interspecific cross (50). The phenotype associated with neither of these mutations seems to be a match with expected tissue impairments of a transglutaminase deficiency. Similarly the pathologies of known congenital human diseases associated with the respective locus are not consistent with a transglutaminase deficiency.

Interestingly, the syntenic region of human chromosome 20q11 is found on distal mouse chromosome 2 adjacent to the...
locus of the identified transglutaminase gene cluster. The genes encoding TGC, and TGE, which are more closely related to the TGM5, TGM7, and EPB42 genes than the other transglutaminases based on amino acid sequence comparison and similarity in gene structure (Fig. 7), have been mapped to human chromosome 20q11 (14, 26). We recently have cloned TGE, which is the product of the TGM6 gene that neighbors the TGM3 gene.4 We have now identified the chromosomal location of the mouse tgm2 and tgm3 genes by radiation hybrid mapping and identified positions ~20 and 5 cM distal to the tgm7/tgm5lep42 gene cluster, respectively. This places a total of six transglutaminase genes on distal mouse chromosome 2 and raises questions about their evolutionary and present-day relationship.

**Evolution of Transglutaminase Genes**—To analyze the evolutionary relationship between the transglutaminase genes in more detail, we calculated the amino acid similarity essentially based on the sequence alignment shown in Fig. 7 and calculated evolutionary distances using different algorithms. All algorithms predicted a close relationship between factor XIII a-subunit and TGK (lineage 1), and TGX, TGY, and vertebrates has been found for many orthologous genes, and vertebrates may have resulted in two successive genome duplications locally to generate the six genes that are clustered on mouse chromosome 2. The exact relationship of TGP to these two lineages is less certain, but it is likely to have branched off from lineage 2 approximately at the same time as factor XIII a-subunit and TGK diverged. Only a single transglutaminase gene has been identified in genomes of invertebrate species, and it is thus likely that the separation of transglutaminases into four branches is likely to have occurred after divergence of invertebrates from proto-vertebrates. In fact, a similar relationship between invertebrates and vertebrates has been found for many orthologous genes, and it has been proposed recently that octaploid in early vertebrates may have resulted in two successive genome duplications in early vertebrates (52, 53). Our phylogenetic analysis is consistent with this model. One branch of lineage 2 has subsequently undergone multiple duplications locally to generate the six genes that are clustered on mouse chromosome 2. Despite the close relationship of neighboring genes within the clusters, one possible scenario is that a single transglutaminase gene initially locally duplicated and was followed by a duplication of a larger segment of the chromosomal region, giving rise to the organization of the genes seen in mouse. In humans these chromosomal regions were apparently redistributed to two different chromosomes. Our analysis further indicates that orthologues of these six genes are likely to exist in all higher vertebrate species.

**Functional Redundancy in Transglutaminase Gene Family**—Despite differential expression in tissues and apparently distinct promoter organization (51, 52), the expression patterns of TGC, TGZ, and TGE overlap.6 Despite differential expression in tissues and apparently distinct promoter organization (51, 52), the expression patterns of TGC, TGZ, and TGE overlap.6 Based on the apparent lack of a phenotype in response to TGX gene ablation in mice, the close similarity of these gene products, and their overlapping expression, we hypothesize that these gene products have overlapping functions.

In conclusion, the identification of several new transglutaminases that are closely related to TGC could explain some of the contradictory data and large number of proposed functions in the literature regarding this enzyme and should stimulate the re-evaluation of these experiments in the light of these findings.

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4. H. Thomas and D. Aeschlimann, manuscript in preparation.
5. D. Aeschlimann and G. Melino, unpublished results.

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**Human TGM5 and TGM7 Genes**

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Evolution of Transglutaminase Genes: Identification of a Transglutaminase Gene Cluster on Human Chromosome 15q15: STRUCTURE OF THE GENE ENCODING TRANSGLUTAMINASE X AND A NOVEL GENE FAMILY MEMBER, TRANSGLUTAMINASE Z

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J. Biol. Chem. 2001, 276:33066-33078.
doi: 10.1074/jbc.M102553200 originally published online June 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102553200

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