Isolation of Markers for Chondro-osteogenic Differentiation Using cDNA Library Subtraction

MOLECULAR CLONING AND CHARACTERIZATION OF A GENE BELONGING TO A NOVEL MULTIGENE FAMILY OF INTEGRAL MEMBRANE PROTEINS* (Received for publication, November 16, 1995, and in revised form, April 9, 1996)

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To identify novel marker molecules associated with chondro-osteogenic differentiation, we have set up a differential screening system based on a cDNA library subtraction in organ cultures of prenatal mouse mandibular condyles. Differential screening of a cDNA library constructed from in vitro cultured condyles allowed the isolation of a novel gene, named E25. Full-length E25 cDNA is predicted to encode a type II integral membrane protein of 263 amino acid residues. In situ hybridization experiments show that E25 is expressed in the outer perichondrial rim of the postnatal mandibular condyle, which contains the proliferating progenitor cells, but not in the deeper layers of the condyle containing the more differentiated chondroblasts and chondrocytes. Other cartilaginous tissues and their perichondrium were negative. Strong in situ hybridization signals were also detected on bone trabeculae of mature bone in tooth germs and in hair follicles. Northern blot analysis showed strong expression in osteogenic tissues, such as neonatal mouse calvaria, paws, tail, and in skin. This expression profile suggests that E25 could be a useful marker for chondro-osteogenic differentiation. Homology searches of DNA databanks showed that E25 belongs to a novel multigene family, containing three members both in man and mouse. The mouse E25 gene locus (Itm2) was mapped to the X chromosome.

It is well established that mesenchymal stem cells can give rise to committed precursors of several lineages of the connective tissue family, including bone and cartilage (1–6). The hierarchy of the different lineages and the mechanism that is involved in their differentiation from the multipotent mesenchymal stem cell have not been completely elucidated. Also there are indications that commitment to one lineage is not necessarily irreversible and that transitions between different cell types may be possible (7–9). In contrast to myogenic (10) and adipogenic (11) commitment, of which the molecular details are gradually being unraveled, the study of the initial stages and molecular control mechanisms of chondro- and osteogenic commitment is less well advanced (12). Although the maturation of preosteoblasts into bone-forming osteoblasts is relatively well known and a model has been proposed, invoking three distinct phases during progressive development of the osteoblast phenotype (13), molecular markers that are specific for the early stages of chondro-osteogenic commitment and differentiation are scarce (6). It is clear that a better understanding of early osteogenic and chondrogenic differentiation would benefit from the availability of a larger number of these early markers.

One strategy to identify marker molecules is to generate monoclonal antibodies that selectively react with various stages of the osteogenic differentiation cascade (14, 15). Other authors have applied subtractive hybridization/differential screening methods (16–21). Most of the differential screening experiments that have been described have focused on systems containing relatively well differentiated osteoblasts. In order to discover additional markers for the early stages of osteogenic differentiation, we have undertaken a differential cDNA screening analysis of organ cultures of prenatal mouse mandibular condyles. The mandibular condyle is a secondary cartilage that, like most elements of the craniofacial skeleton, arises from neural crest-derived mesenchyme (22). In vivo, mesenchymal-like stem cells in the perichondrium give rise to skeletal precursor cells (skeletoblasts) which enter the chondrogenic differentiation pathway and eventually undergo enchondral ossification. However, when the perinatal condyle is explanted and cultured in organ culture, the perichondrial precursor cells enter osteogenic differentiation, start expressing bone matrix-specific markers, and form a mineralized bone matrix over a period of 2–3 weeks (23–26). Since the condylar precursor cells develop exclusively along the chondrogenic pathway in vivo and are induced only by culture conditions to switch to osteogenic differentiation, condyle organ cultures constitute a suitable experimental system in which to study and isolate early markers of bone differentiation.

In this study we have subtracted cDNA from 1-day-old condyle explant cultures with an excess driver RNA derived from the fibroblastic cell line Balb/3T3 (27). This subtracted cDNA was used as a probe to differentially screen a cDNA library from 1-day-old explanted condyles. We preferred to use Balb/3T3 RNA as a source of subtractor RNA rather than RNA from uncultured condyles (t = 0) as this would allow for simultaneous isolation of novel chondrogenic markers, which can be
assumed to be still present in condylar tissue after 1 day of culture. At the same time the Balb/3T3 cell line is relatively close to the chondro- and osteogenic lineages in order to allow subtraction of a maximum of lineage-nonspecific genes. In view of the limiting amounts of RNA that could be obtained only from fetal condyles and which would preclude performing multiple subtractive hybridizations, we developed a subtractive hybridization/differential screening approach in which subtracted cDNA could be repeatedly generated from target (1-day-old condyle explants) and driver (Balb/3T3) cDNA libraries. Rather than converting the subtracted cDNA in a subtracted cDNA library, which is generally of poor quality and needs further sub screening to identify differentially expressed transcripts, the subtracted cDNA was used as a probe in conjunction with unsubtracted cDNA to differentially screen the 1-day-old explant condyl e cDNA library. This has the advantage that target specific transcripts which are thus enriched in the subtracted probe can be immediately identified and isolated.

After screening a limited number of clones with this approach, we have been able to isolate a novel gene, which shows strong although not exclusive expression in osteogenic (but not chondrogenic) tissue, and belongs to a previously not described multigene family.

MATERIALS AND METHODS

Mandibular Condyle Organ Culture/Cell Culture—Pregnant Balb/c mice were anesthetized with ether at 18.5 days post coitum and sacrificed by cervical dislocation. Fetuses were removed and cooled on ice. Fetal mandibular condyles were dissected and cleaned from adherent soft tissues. Extra care was taken to remove traces of mandibular or endochondral bone. Condyles were then transferred onto collagen sponges (Porous Collagen Matrices M1531, American Biomaterials Corp., Plainsboro, N.J.) which were placed on stainless steel grids at the medium (BGJ/b with 10% fetal bovine serum) (95% air, 5% CO2) interface of a 37°C incubator (at 100% humidity). Six to eight condyles were cultured per sponges in six-well plates. To construct the t = 1 day cDNA library, approximately 750 condyles were placed in culture, and batches of about 100 condyles were harvested at 1-h intervals between 18 and 24 h and subsequently pooled. Freshly isolated or in vitro cultured condyles were immediately frozen in liquid nitrogen until RNA extraction. Balb/3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum until subconfluency, RNA was extracted from condyles or from Balb/3T3 cell pellets according to the procedure of Chirgwin et al. (28). About 0.25 μg of total RNA was obtained per condyle. Poly(A)+ RNA was prepared by two sequential rounds of affinity selection using oligot(dT)24 DynabeadsTM (Dynal A.S., Oslo, Norway), according to the protocol recommended by the supplier.

Construction of the Primary cDNA Libraries—Directional cDNA libraries of in vitro cultured condyles (t = 1 day) and the Balb/3T3 cell line were constructed using the SuperscriptTM Plasmid system (Life Technologies, Inc.). cDNA was size-fractionated on a Sephacryl S-500 column and electrophoresed into Escherichia coli Sure TM (Stratagene) bacteria. For the Balb/3T3 and condyle t = 1 day cDNA libraries, approximately 1.3 × 105 and 2.3 × 104 independent colonies were collected, respectively. Plasmid DNA was prepared from the pooled bacteria using QiaGen™ columns (Qiagen GmbH, Germany). Insert sizes of 20–25 random clones from each library were determined. The average insert sizes were 0.9 and 1.8 kb for the Balb/3T3 and condyle t = 1 day libraries, respectively. The number of empty clones, as assessed by blue-white screening in the presence of isopropyl-1-thio-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside, was only a few percent in both libraries.

Subcloning of the Condyle t = 1 Day cDNA Library—Plasmid DNA from the condyle t = 1 day pSPORT1 library was NotI/Sall-digested and separated on a preparative 0.9% agarose gel. The cDNA smear between 0.5 and 4 kb was purified from the gel using the GenecleanTM kit (BIO 101 Inc., Vista, CA). This cDNA was then sub cloned into two different vectors (see Fig. 1). (i) pGEM9Zf+; purified c DNA was ligated into NotI/Sall-digested pGEM9Zf+ (Promega, Madison, WI) and electroporated into E. coli DH12S (Life Technologies, Inc.). The sub cloned pGEM9Zf+ library contained 18 × 104 independent clones, 91% of which contained an insert. Plasmid DNA was prepared from an aliquot of the collected bacteria. (ii) λgt10 for sub cloning into the λgt10 vector, PCR amplified fragments were first blunted with T4 DNA polymerase, ligated to EcoRI adaptors using the RiboclonTM EcoRI adaptor ligation system (Promega) and finally digested with HindIII. This digestion served to render contaminating NotI/Sall linearized pSPORT1 vector molecules unclam able at the expense of a small proportion of the insert fragments which happen to contain an internal HindIII site. Inserts were then ligated into λgt10 EcoRI arms. The λgt10 library consisted of 50,000 independent clones, 75% of which contained an insert.

Preparation of Driver and Target cRNA—Driver and target cDNA were generated by in vitro transcription using the MegascriptTM system for large scale RNA synthesis (Ambion, Austin, TX) according to the instructions recommended by the supplier. The procedure was divided into three steps. (i) Firstly, cDNA clones were annealed (t = 70°C) to 1 μg of random primers (mostly hexamers; Life Technologies, Inc.) and reverse transcribed for 1 h at 37°C with 1,000 units of SuperscriptTM (Life Technologies, Inc.) reverse transcriptase in a total volume of 20 μl (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 500 μM each dNTP). One μl of [α-32P]cATP (American Corp., specific activity 3,000 C/mmoll) was added to the reaction to allow accurate quantification. The reaction was stopped by adding EDTA to a final concentration of 50 mM, and the cRNA template was hydrolyzed by incubation in 0.2 N NaOH (20 min at 65°C). The solution was neutralized by adding 20 μl of 1 M Tris, pH 7.5, and 20 μl of 1 M HCl, and the cDNA was purified by spin chromatography on Sephadex G-50 (Pharmacia Biotech Inc.) in the presence of SDS (0.5%) and stored at −70°C until used.

Subtractive Hybridi zations—For subtractive hybridization reactions 2–4 μg of first strand cDNA, generated from the = 1 day cDNA library as described above, were spiked with 1–5 ng each of P. chabaudi clones 100 and 110 first strand cDNA (see Fig. 1). A fraction of this cDNA was used as unsubtracted probe while the rest was mixed with 100 μg of biotinylated Balb/3T3 driver cDNA and equilibrated in 10 mM Hepes, pH 7.5, 0.4 mM EDTA, 0.04% SDS by ultrafiltration in a microcon 10 (Ammco) concentrator to a final volume of 100 μl. The solution was then hybridized to a concentrated solution of 18S ribosomal RNA and finally 2 μl of 5 mM NaCl were added. The reaction mixture was overlaid with mineral oil, heated for 5 min in a boiling water bath, and immediately transferred to a 65°C water bath for 15–24 h (achieving a Rot of 800–1200 mol L−1 s−1). The final composition of the hybridization reaction was 50 mM Hepes, pH 7.5, 2 mM EDTA, 0.2% SDS, 0.5 mM NaCl (hybridization buffer), and 5 μg/ml driver biotin cDNA. After the hybridization was completed, 80 μl of hybridization buffer without SDS were added to the reaction. The mineral oil was removed by CHCl3 extraction. Sub tractions were done by incubating the hybridization mix with 20 μg of streptavidin for 15 min at room temperature and then extracting with phenol/CHCl3 (1:1) (29). Subtractions were repeated until no more counts could be detected in the organic phase (usually three to four rounds). The concentration of the remaining cDNA was determined by scintillation counting.

Differential Screening—Three sequential plaque lifts onto Hybond-N membranes (Amersham) were taken. The first lift was discarded, whereas the second and third lift were hybridized to the unsubtracted
and subtracted probe, respectively. Preliminary experiments had shown that there was virtually no difference in signal intensity of individual plaques between lifts 2 and 3 when these were hybridized to the unsubtracted (complex) probe. Transferred DNA was UV-cross-linked, and membranes were hybridized for 24 h to 25 ng of random primed labeled subtracted or unsubtracted cDNA in 5 ml of Rapid HybTM solution (Stratagene) at 65 °C. Specific activities of subtracted and unsubtracted probes did usually not differ from each other by more than a few percent and were usually greater than 10^9 dpm/µl. After hybridization, filters were washed two times for 15 min in 2 × SSC, 0.5% SDS at room temperature and finally two times for 4 h in 0.1 × SSC, 0.5% SDS at 65 °C. Differentially hybridizing plaques were isolated, replated, and subjected to a second round of differential screening.

Northern Blot Analysis and 5' - RACE—RNA samples were separated on denaturing formaldehyde gels, blotted, and hybridized according to standard procedures (30). Blots were washed to a final stringency of 1 × SSC, 0.1% SDS at 42 °C.

Determination of the 5’-end of the E25 transcript was done using the 5’-RACE system (Life Technologies, Inc.), using the nested E25 antisense primers complementary to positions 1397–1416 and 1099–1118, respectively (see Fig. 3).

Analysis of Expressed Sequence Tag (EST) Sequences—EST sequences were identified from the dbEST DNA database using the BLAST algorithm (31). The E2S4HS sequence was obtained by merging the deduced amino acid sequences of human ESTs D65046, H38038, H50965, R01594, R01595, R50284, R57161, T93061, and T93146. The E25BHS sequence was obtained via merging the DNA sequences of the following human ESTs: D56480, D56598, D56653, D56674, D56726, D56727, D56797, D56994, D57095, D57146, D57278, D57740, D57751, D57790, D57913, D57976, D58336, D58342, F11245, H02842, H25679, H43936, M78850, R11871, R56705, R62686, R63001, R64398, R66830, R76391, R76729, R80097, R80098, R86131, T05330, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35799, T39406, Z16297, and Z24337. The deduced amino acid sequence of the merged consensus DNA sequence was named E25BHS. The (partial) E25CHS amino acid sequence was obtained from merging the deduced amino acid sequences of the following human ESTs: H23306, H53246, R35030, R66500, R66830, R76391, R76729, R80097, R80098, R86131, T05330, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486, T07918, T30142, T30402, T30699, T30915, T31525, T31755, T34032, T34043, T34119, T34830, T35254, T35344, T35679, T35861, T39262, T39616, T39622, T39629, T39638, T63294, T93743, T93745, T93748, T94486.
E25: a Novel Marker Associated with the Osteogenic Phenotype

TABLE I
Evaluation of the subtracted probes

| Test sequence                  | Hybridization signal ratio (subtracted/unsubtracted probe) |
|--------------------------------|------------------------------------------------------------|
| Mouse α1(I) procollagen        | 0.31                                                       |
| Mouse α2(I) procollagen        | 2.10                                                       |
| Mouse α1(IX) procollagen       | 5.16                                                       |
| P. chabaudi clone 100          | 2.01                                                       |
| P. chabaudi clone 110          | 2.87                                                       |
| Human osteonectin              | 0.30                                                       |
| Mouse GAPDH                    | 0.30                                                       |
| Mouse TGF-β                     | 0.83                                                       |
| Mouse β-actin                  | 0.28                                                       |

The complex probe consisted of first strand cDNA which was generated from the condyle t = 1 day pGEM9zf′ library and “doped” with heterologous first strand cDNA (P. chabaudi clones 100 and 110). Part of this probe was used as the unsubtracted control, whereas the rest was subtracted with an excess of Balb/3T3 bio-cRNA. The Southern blots contained different recombinant plasmids which had been restriction digested to release the inserts. Hybridized insert bands were cut from the blot and quantitated via scintillation counting. For each test insert the ratio of the hybridization signal obtained with the subtracted probe (average of two Southern blots using the same probe preparation) to that of the unsubtracted probe is indicated.

Using this modified strategy, subtracted probe was prepared by subtracting cDNA from the condyle t = 1 day pGEM9zf′ library with biotinylated cRNA generated from the Balb/3T3 pSPORT1 library. The quality of the subtracted probes was evaluated by differential hybridization on Southern blots containing a number of test sequences. Bands containing the test sequences were cut from the hybridized blot and quantitated via scintillation counting. Table I shows that the subtractive hybridization was specific. Sequences that are clearly known to be expressed in both fibroblastic and condylar tissue (e.g. collagen I, osteonectin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin) showed decreased hybridization with the subtracted probe, indicating that they had indeed been partially removed. In contrast, sequences that are known to be specific for the chondrogenic tissue (collagen II, collagen IX) or exogenously added unrelated DNA sequences (P. chabaudi clones 100 and 110) showed increased hybridization signals with the subtracted probe and thus had been selectively enriched. For transforming growth factor-β, the difference in hybridization signal between subtracted and unsubtracted probe was less pronounced than for the other test sequences.

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Differential Screening of the Condyle t = 1 Day cDNA Library—Approximately 5,000 plaques of the condyle t = 1 day cDNA library were screened with both unsubtracted and subtracted probes on duplicate plaque lifts. 25 plaques (E1–25) that hybridized more strongly to the subtracted than to the unsubtracted probe were isolated. 10 clones could be confirmed after a second round of differential screening and were partially sequenced. Comparison of the obtained sequence information with DNA databanks showed that all sequences except E22 and E25 had been described previously. The known sequences consisted of H19 (three clones), collagen II (three clones), bone sialoprotein (one clone), and placental alkaline phosphatase (one clone). Although clone E22 did not match any sequence present in DNA databanks, its high degree of similarity to human collagen IX suggested that it encoded mouse collagen IX. The presence of collagen II, collagen IX, and bone sialoprotein among the clones that were enriched after condyle

minus Balb/3T3 subtraction is not unexpected as all of these genes are known to be expressed in cartilagenous tissue such as the condyle and not in the Balb/3T3 cell line (34, 35). In addition we confirmed by Northern blot analysis that the H19 transcript was indeed expressed in condylar RNA (1-day explants) but was absent in Balb/3T3 RNA (data not shown). Likewise, a radiolabeled E25 probe strongly hybridized to a band of ±1.8 kb on condylar RNA but did not hybridize to Balb/3T3 RNA (Fig. 2A). It was thus clear that all clones isolated after differential screening were present in the target tissue and absent in the Balb/3T3 driver population, further demonstrating the reliability of the subtractive screening procedure. We decided to characterize the novel clone E25 in greater detail.

Northern Blot Analysis—Northern blot analysis of different mouse neonatal tissues showed very strong expression of E25 in calvaria and slightly weaker expression levels in front paws, skin, and tail (Fig. 2B). Expression in brain and heart was very weak, whereas there was no detectable transcript in liver and lung.

Analysis of the E25 Primary Sequence—Two additional clones were isolated from the condyle pSPORT1 cDNA library, which extended the known E25 sequence by 200 bp at the 5′ end. 5′-RACE analysis on mRNA from freshly isolated condyles led to the isolation of five more clones, again extending the E25 sequence at the 5′ end with 50 bases. The composite nucleotide sequence obtained from these overlapping clones was 1654 bp in length and contained a polyadenylation signal followed by a poly(A) tail at its 3′ end (Fig. 3). The cDNA contained a single long open reading frame (nucleotides 1–939). The presumed initiator methionine occurred at position 151–153. Several arguments underscored this view. (i) All five clones obtained by 5′-RACE analysis stopped within a window of 5 base pairs at their 5′ end, suggesting that the reverse transcriptase had indeed reached the mRNA start point. (ii) The size of the obtained E25 cDNA was very close to the size of the transcript...
produce a protein of 263 amino acids with a translation initiation codon (ATG) in front of the initiator codon in question. It is therefore likely that their consensus sequence contains an in-frame stop codon equivalent (see below) that are available in this region shows homology with E25AHS (R01594, R50284, T93146, and R57511) from the human E25 protein. The putative transmembrane domain is marked with a double underline, and the polyadenylation signal is in bold. Inspection of four EST sequences (E25AHS and E25BHS) will refer to the mouse gene isolated in this study as E25AMM. The "ATTTA" sequence motif in the 3'-untranslated region have been underlined. The (iso)leucine residues in the potential leucine zipper motif are in bold and crosses, whereas the single R1 domain linked glycosylation site is indicated by *. The (iso)leucine residues in the potential leucine zipper motif, motif analysis did not reveal any structural features of known protein families. Overall the E25 protein has a high content of leucine (10.2%) and isoleucine residues (8.0%). The E25 mRNA has a rather long 3'-untranslated region of ±700 bp, containing five “ATTTA” sequence motifs, which have been shown to mediate RNA destabilization in certain lymphokine and immediate early genes (40, 41).

In Situ Hybridization Experiments—In order to study expression of E25 mRNA in more detail, in situ hybridization experiments were carried out on frozen sections of neonatal mice (Fig. 4). In the neonatal mouse mandibular condyle strong ISH signals were observed only in the outermost layers, corresponding to the perichondral and subperichondrial region. There was a marked absence in the deeper layers of the condyle, which contain the more differentiated chondroblasts and chondrocytes. This could not be due to an artefactual loss of mRNA from the deeper layers of the condyle as a result of section preparation, because under the same conditions, we were able to obtain strong ISH signals throughout the condylar tissue with another clone derived from the mandibular condyle. Prominent ISH signals were also detected on the trabeculae of various bones including the ossified parts of ribs. In contrast, E25 ISH signals were markedly absent in the cartilagenous parts of ribs, vertebrae, and long bones. Interestingly, strong expression of E25 was also observed in mature odonto-blasts of germinating teeth but not in the less differentiated mesenchyme of the dental papilla. Hair follicles and stratum corneum of the skin also stained strongly positive. E25 was strongly expressed in the acini of several exocrine glands such as parotid gland, lacrimal gland, and seromucous glands in nose mucosa. Finally weak ISH signals were found in the brain (choroid plexus), renal cortex, and in the crypts of the small intestine.

 Establishment of the E25 Multigene Family—Homology searches of DNA databanks using the BLAST algorithm showed that more than 80 ESTs of human and murine origin had been deposited, of which the open reading frame showed significant homology to the E25 protein sequence. Detailed analysis of these ESTs revealed that E25 belongs to a multi-gene family which contains at least three members (Fig. 5). We will refer to the mouse gene isolated in this study as E25AMM (E25A Mus musculus). Via alignment of a subset of the human ESTs (nine sequences) we were able to obtain the complete coding sequence of the human equivalent of E25AMM, which we named E25AH (E25A Homo sapiens). The amino acid sequences of E25AMM and E25AH are 94% identical. The majority of the human ESTs (63 sequences) could be aligned to yield a full-length protein sequence, which was 38% identical to E25AH and was named E25BHS. The deduced amino acid sequence of murine EST L26775 was nearly identical to E25AHS and was named E25BHS. The deduced amino acid sequence of murine EST L26775 was nearly identical to E25BHS (93%) suggesting that it represents the murine counterpart, which we named E25BHS. Finally nine human EST sequences could be aligned to yield a full-length protein sequence, which was 38% identical to E25AH and was named E25BHS. The deduced amino acid sequence of murine EST L26775 was nearly identical to E25BHS (93%) suggesting that it represents the murine counterpart, which we named E25BHS. Finally nine human EST sequences could be aligned to yield a partial protein sequence (131 amino acids) which was related to but distinct from the E25BHS sequence. The deduced amino acid sequence of murine ESTs R75511, R74770, and R75509 was 91% identical to the E25BHS partial amino acid sequence, suggesting that these ESTs encode the murine counterpart of E25BHS, referred to as E25CMM.

 Conservation was less pronounced between different isoforms within the same species. For instance E25AH was 38% identical to E25BHS and 49% to E25CMM (it should be noted that in contrast to E25BHS and E25CMM, the E25CHS sequence information was only partial). The ESTs representing E25AH were derived from fetal brain, heart, and liver/spleen, 2 G. Hong, unpublished observations.

2 G. Hong, unpublished observations.

Fig. 3. Full-length cDNA sequence and predicted protein sequence of E25AMM. The initiator and terminator codons are in bold. The putative transmembrane domain is marked with a double underline, whereas the single N-linked glycosylation site is indicated by *. The (iso)leucine residues in the potential leucine zipper motif are in bold and crosses, whereas the single R1 domain linked glycosylation site is indicated by *. The (iso)leucine residues in the potential leucine zipper motif, motif analysis did not reveal any structural features of known protein families. Overall the E25 protein has a high content of leucine (10.2%) and isoleucine residues (8.0%). The E25 mRNA has a rather long 3'-untranslated region of ±700 bp, containing five “ATTTA” sequence motifs, which have been shown to mediate RNA destabilization in certain lymphokine and immediate early genes (40, 41).

In Situ Hybridization Experiments—In order to study expression of E25 mRNA in more detail, in situ hybridization experiments were carried out on frozen sections of neonatal mice (Fig. 4). In the neonatal mouse mandibular condyle strong ISH signals were observed only in the outermost layers, corresponding to the perichondral and subperichondrial region. There was a marked absence in the deeper layers of the condyle, which contain the more differentiated chondroblasts and chondrocytes. This could not be due to an artefactual loss of mRNA from the deeper layers of the condyle as a result of section preparation, because under the same conditions, we were able to obtain strong ISH signals throughout the condylar tissue with another clone derived from the mandibular condyle. Prominent ISH signals were also detected on the trabeculae of various bones including the ossified parts of ribs. In contrast, E25 ISH signals were markedly absent in the cartilagenous parts of ribs, vertebrae, and long bones. Interestingly, strong expression of E25 was also observed in mature odonto-blasts of germinating teeth but not in the less differentiated mesenchyme of the dental papilla. Hair follicles and stratum corneum of the skin also stained strongly positive. E25 was strongly expressed in the acini of several exocrine glands such as parotid gland, lacrimal gland, and seromucous glands in nose mucosa. Finally weak ISH signals were found in the brain (choroid plexus), renal cortex, and in the crypts of the small intestine. Striated muscle, liver, and lung showed no E25 expression. Sense probes were completely negative on all sections (data not shown).

Establishment of the E25 Multigene Family—Homology searches of DNA databanks using the BLAST algorithm showed that more than 80 ESTs of human and murine origin had been deposited, of which the open reading frame showed significant homology to the E25 protein sequence. Detailed analysis of these ESTs revealed that E25 belongs to a multi-gene family which contains at least three members (Fig. 5). We will refer to the mouse gene isolated in this study as E25AMM (E25A Mus musculus). Via alignment of a subset of the human ESTs (nine sequences) we were able to obtain the complete coding sequence of the human equivalent of E25AMM, which we named E25AH (E25A Homo sapiens). The amino acid sequences of E25AMM and E25AH are 94% identical. The majority of the human ESTs (63 sequences) could be aligned to yield a full-length protein sequence, which was 38% identical to E25AH and was named E25BHS. The deduced amino acid sequence of murine EST L26775 was nearly identical to E25AHS and was named E25BHS. The deduced amino acid sequence of murine EST L26775 was nearly identical to E25BHS (93%) suggesting that it represents the murine counterpart, which we named E25BHS. Finally nine human EST sequences could be aligned to yield a partial protein sequence (131 amino acids) which was related to but distinct from the E25BHS sequence. The deduced amino acid sequence of murine ESTs R75511, R74770, and R75509 was 91% identical to the E25BHS partial amino acid sequence, suggesting that these ESTs encode the murine counterpart of E25BHS, referred to as E25CMM.

 Conservation was less pronounced between different isoforms within the same species. For instance E25AH was 38% identical to E25BHS and 49% to E25CMM (it should be noted that in contrast to E25BHS and E25CMM, the E25CHS sequence information was only partial). The ESTs representing E25AH were derived from fetal brain, heart, and liver/spleen, 2 G. Hong, unpublished observations.
and from adult lung and breast. E25BHS ESTs originated from a variety of tissues including fetal brain and liver/spleen and from aorta, placenta, lung, prostate, adrenal gland, white blood cells, and endothelial cells. E25CHS ESTs were derived from brain, placenta, and fetal liver/spleen.

Chromosomal Mapping—The radiolabeled E25AMM probe hybridized with a 4.5-kb BglII DNA fragment from strain C57BL/6 and a 4.3-kb BglII DNA fragment from the SEG strain. This size polymorphism was used to localize the E25AMM locus (which we named Itm2, integral membrane protein 2, as a successor to Itm1, the gene for another integral membrane protein, that we characterized previously) by linkage analysis. We found that all the DNA samples derived from the male offspring of the backcross were all of one parental type, either C57BL/6 or SEG. Such an observation indicated X-linkage. Checking the allelic distribution of E25 for the 18 females, we found no recombinant with two X-linked loci: DXMit8 and Xist.

**DISCUSSION**

We have used differential cDNA screening of organ cultures of prenatal mouse mandibular condyles to identify and isolate a gene encoding a novel integral membrane protein, E25AMM, that is differentially expressed in bone and cartilage tissue and belongs to a multigene family.

We developed an alternative subtractive hybridization/differential screening protocol, which allowed us to directly use library derived subtracted cDNA as a probe in differential screening, a strategy which to our knowledge has not been described so far in the literature. We found that the major difficulty in using library derived cDNA as a probe for differential screening, was that cDNA probes, which are generated from libraries by sequential in vitro transcription and reverse transcription are contaminated with vector derived sequences. We solved this problem by generating subtracted probes from a plasmid library and using these probes in the differential screening of a cDNA library made in the unrelated λgt10 vector.

The reliability of the subtractive hybridization protocol was investigated. We found that cDNAs known to be expressed in both condylar tissue and fibroblasts (GAPDH, β-actin, and α1(I) collagen) were indeed subtracted. Quantitative analysis showed that the hybridization signal of the subtracted genes in the subtracted probe was around 30% of that in the unsubtracted probe. It should, however, be noted that equal amounts of both subtracted and unsubtracted cDNA were used in the differential screening procedure. Because one round of subtraction typically removed 60–80% of the cDNA, the hybridization signal of subtracted species was artificially increased. When one corrects for these effects, it becomes clear that over 90% of each of the above-mentioned cDNAs has been removed. On the other hand, genes which are known to be specifically expressed in neonatal mouse condyles such as the cartilage specific α1(II) and α1(IX) collagen cDNAs as well as the two exogenously added P. chabaudi cDNA species had an increased abundance in the subtracted probe. The increase in abundance ranged from a factor 2.0 (P. chabaudi clone 100) to 5.16 (α1(IX) collagen). Theoretically the maximal increase in abundance is dictated by the total amount of cDNA that can be subtracted and thus by the degree of similarity between the two RNA populations. The fact that typically 60–80% of the cDNA is removed after subtraction is in agreement with the experimentally observed increase in hybridization intensity of unsubtracted
cDNA species. It is not clear why some cDNAs are more enriched than other ones. Possibly there is some degree of aspecific subtraction of cDNA based on accidental low level homology between cRNA in the driver and some target cDNA molecules. The enrichment factor we observe is comparable to that reported in other systems (43–46). Although substantially higher enrichment factors have been reported by some authors it is not always clear whether these results had been corrected for yield losses during the subtractive hybridization procedure or whether other artefacts could explain the high subtraction efficiencies, as remarked in a recent review (47).

The identity of the 10 differential clones that were isolated further demonstrated the specific character of the subtractive hybridization/differential screening protocol. Bone sialoprotein, collagen II, and collagen IX are indeed known to be specifically expressed in the cartilagenous tissue of the mandibular condyle but not in Balb/3T3 fibroblasts. Furthermore, Northern blot analysis showed that the H19 and E25 transcripts were completely absent in Balb/3T3 fibroblasts, but strongly expressed in mandibular condyles. The H19 transcript is a noncoding RNA molecule that is expressed in many fetal tissues and which was shown to have tumor suppressor activity (48).

An intriguing finding was the presence of placental alkaline phosphatase in mandibular condyles. Whereas a potential contamination of the fetal condyle tissue with placental material during isolation, although unlikely, cannot be excluded, these results raise the possibility that fetal condyles express the placental isotype of alkaline phosphatase instead of the liver/placental isotype which is generally found in cartilagenous tissue.

The aim of the current experiments was to identify novel markers of osteogenic differentiation. Our results indicate that E25 could be a useful marker in this respect. In situ hybridization experiments showed that E25 expression in neonatal mandibular condyles was clearly limited to the outer rim of the condyle, which contains the prechondroblastic mesenchymal cells. All other cartilagenous tissues that were examined by in situ hybridization (ribs, long bones, vertebrae) were negative for E25. By contrast there was strong staining of bone trabeculae in mature bone. A strong association of E25 with osteogenic tissue was also suggested by the Northern blot analysis of various neonatal mouse tissues. By far the strongest expression was observed in calvaria and to a lesser extent also in front paws and tail. These tissues, especially calvaria, are actively involved in osteogenesis. With the exception of skin, expression in nonosteogenic tissues was weak (brain and heart) or absent (lung and liver).

We hypothesize that expression of the E25 marker in the precursor cells of the mandibular condyle reflects the ontogeny of this secondary cartilage. Avian secondary cartilages develop from the periosteal cells of membrane bones (49), whereas mammalian mandibular condyles originate as a separate cellular blastema and later associate with the membranous bone of the mandible (50, 51). The initial blastemal type of development is thus succeeded by a periosteal/perichondrial type of development. The E25 marker which we showed to be strongly expressed in mature osteoblasts would thus be gradually turned off in the differentiating periosteum/perichondrium of the condyle during secondary chondrogenesis. This would also explain why the perichondrium of primary cartilages (e.g. rib) is negative, as it does not differentiate from membranous bone.

Although E25 expression is strongly associated with mature osteoblasts and the early stages of (secondary) chondrogenesis in the mandibular condyle it appears not to be uniquely restricted to these tissues. This is shown by the weak Northern blot signals that were detected in (fetal) heart and brain and the relatively strong signal in skin. Strong in situ hybridization signals were indeed found in hair follicles and stratum corneum of the skin. The acini of several exocrine glands also exhibited strong ISH staining. Weaker ISH signals were also detected in brain, renal cortex and in the small intestine.

It is unlikely that the positive ISH signals are due to crosshybridization to other members of the E25 family. Although complete sequence information is not available on all three mouse variants, it is clear that even in the more conserved C-terminal part the difference in sequence between different members is large enough for the melting temperature of crosshybrids to be substantially lower than that of the specific hybrid. Also if there was some cross-hybridization, this would have been detected on the Northern blots, where additional bands or at least a more diffuse band would have been present for the embryonic tissues that were investigated. However, only a single discrete band was present on the Northern blots (Fig. 2).

The E25AMM predicted amino acid sequence contains a single putative transmembrane domain between amino acids 52–76 and no amino-terminal signal peptide, suggesting that it is a type II integral membrane protein with the C-terminal part being extracellular. This topology would fit with the position of the sole potential N-glycosylation site (Asn-166). The significance of the leucine zipper motif is unclear. Whereas leucine zipper motifs are well known as protein-protein interaction domains in transcription factors, they presumably also mediate multimerization in other classes of proteins. Alternatively it could be argued that the observed leucine zipper is purely accidental, due to the high leucine/iso-leucine content of the E25 protein.

DNA databank searches yielded a multitude of human and murine EST sequences, which were homologous to the
E25AMM sequence. From these sequences a E25 multigene family, containing three members both in mouse and man, emerged. Individual family members exhibited a very high degree of conservation between man and mouse, whereas the homology between the different family members was around 40% within the same species. All three members appear to be integral membrane proteins since they all contain a single transmembrane domain (roughly between amino acid 50 and 70). Individual members show the highest degree of conservation in the amino-terminal (presumably cytoplasmic) part. The leucine zipper motif present in E25A (both in man and mouse) was not retained in the E25B and E25C members. Like in E25A, no distinct motifs of known protein families could be detected in the available parts of the E25B and E25C protein sequences.

Judging from the origin of the cDNA libraries from which E25 related ESTs have been isolated, members of the E25 gene family are expressed in a wide variety of tissues and some tissues appear to be capable of expressing all three members (eg. brain). Whether the E25B and E25C family members are also associated with osteogenic/chondrogenic differentiation will need further experimentation.

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