Transcription Factor ERG Variants and Functional Diversification of Chondrocytes during Limb Long Bone Development

Masahiro Iwamoto,* Yoshinobu Higuchi,* Eiki Koyama,* Motomi Enomoto-Iwamoto,* Kojiro Kurisu,* Helena Yeh,§ William R. Abrams,§ Joel Rosenbloom,§ and Maurizio Pacifici§

*Department of Oral Anatomy and Developmental Biology and ‡Department of Biochemistry, Osaka University Faculty of Dentistry, Osaka 565, Japan; and §Department of Anatomy and Histology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6003

Abstract. During limb development, chondrocytes located at the epiphyseal tip of long bone models give rise to articular tissue, whereas the more numerous chondrocytes in the shaft undergo maturation, hypertrophy, and mineralization and are replaced by bone cells. It is not understood how chondrocytes follow these alternative pathways to distinct fates and functions. In this study we describe the cloning of C-1-1, a novel variant of the ets transcription factor ch-ERG. C-1-1 lacks a short 27–amino acid segment located ~80 amino acids upstream of the ets DNA binding domain. We found that in chick embryo long bone anlagen, C-1-1 expression characterizes developing articular chondrocytes, whereas ch-ERG expression is particularly prominent in prehypertrophic chondrocytes in the growth plate. To analyze the function of C-1-1 and ch-ERG, viral vectors were used to constitutively express each factor in developing chick leg buds and cultured chondrocytes. We found that virally driven expression of C-1-1 maintained chondrocytes in a stable and immature phenotype, blocked their maturation into hypertrophic cells, and prevented the replacement of cartilage with bone. It also induced synthesis of tenascin-C, an extracellular matrix protein that is a unique product of developing articular chondrocytes. In contrast, virally driven expression of ch-ERG significantly stimulated chondrocyte maturation in culture, as indicated by increases in alkaline phosphatase activity and deposition of a mineralized matrix; however, it had modest effects in vivo. The data show that C-1-1 and ch-ERG have diverse biological properties and distinct expression patterns during skeletogenesis, and are part of molecular mechanisms by which limb chondrocytes follow alternative developmental pathways. C-1-1 is the first transcription factor identified to date that appears to be instrumental in the genesis and function of epiphyseal articular chondrocytes.

Key words: transcription factor ERG • articular chondrocytes • growth plate chondrocytes • limb development • tenasin-C

Introduction

During limb skeletogenesis, chondrocytes follow two distinct developmental pathways to two distinct fates and functions. The relatively few chondrocytes present at the epiphyseal extremity of long bone anlagen develop into permanent articular chondrocytes, which give rise to articular cartilage, produce abundant extracellular matrix, and maintain normal joint function throughout life (Mitrovic, 1977, 1978; Archer et al., 1994; Pacifici, 1995). In contrast, the much more numerous chondrocytes constituting the shaft of the long bone anlagen are transient, become organized into growth plates, undergo maturation, hypertrophy, mineralization and apoptosis, and are eventually replaced by bone cells via endochondral ossification (Howlett, 1979; Hunziker, 1994). Both populations of chondrocytes are of obvious crucial importance for skeletogenesis and skeletal function. By organizing and maintaining the articular tissue, articular chondrocytes allow the joints and associated structures to exert their important biomechanical roles and permit normal body movements throughout life. Likewise, by undergoing proliferation, maturation, and hypertrophy, the growth plate chondrocytes allow each skeletal element to grow in...
length and width, contribute to the determination of shape and orientation of the elements, and permit the normal replacement of hypertrophic mineralized cartilage with definitive bone and bone marrow.

Given the fundamental roles played by articular and growth plate chondrocytes in skeletal organization and function, it is not surprising that there is ever increasing interest in them. This has led to the identification of a number of molecules with important roles in the development, behavior, and function of growth plate chondrocytes, including signaling molecules, growth factors, and transcription factors (Colvin et al., 1996; Vortkamp et al., 1996; Enomoto-Iwamoto et al., 1998; Koyama et al., 1999). In contrast, we remain largely ignorant about the mechanisms leading to the development of articular chondrocytes, particularly at the molecular level. Thus, we know little about how the numerically few presumptive articular chondrocytes emerging at the epiphyseal tip of long bone anlagen: (a) acquire their permanent phenotype, (b) escape the maturation and ossification process followed by the more numerous growth plate chondrocytes, (c) transmit these properties to their progeny, and (d) remain functional throughout life. Detailed information on articular chondrocyte development and function would not only be of intrinsic biological interest, but is also likely to have important medical ramifications for joint diseases such as osteoarthritis, in which the articular chondrocyte phenotype is altered and joint function is progressively lost (Hamer, 1989).

It was shown recently that the transcription factor ERG is expressed at sites of future joint development in the early chick limb (Ganan et al., 1996; Macias et al., 1997). ERG belongs to the ets family of transcription factors, which are highly conserved and are involved in a variety of biological processes (Ra et al., 1987; Wasylyk et al., 1993, 1998). Every family member contains the ets domain and function is progressively lost (Hamer, 1989).

Materials and Methods

Cartilage Tissue Isolation and RT-PCR

Samples of articular cartilage layer and underlying growth plate were microsurgically isolated from day 17 chick embryo tibiotarsus or femurs. Care was taken to remove adhering perichondral and connective tissues and obtain as homogeneous cartilaginous tissue as possible. RNA was isolated by the guanidium isothiocyanate method (Iwamoto et al., 1993b) and processed for standard RT-PCR as described (Enomoto-Iwamoto et al., 1998). In brief, 1 μg of RNA was reverse transcribed with Superscript reverse transcriptase (GIBCO BRL) and random hexamers, and amplification was carried out with E longa (GIBCO BRL) for 30 cycles (95°C for 10 s and 60°C for 1 min) with the following ch-ERG sense and antisense primers (indicated as primer A and primer B in Fig. 1 B, respectively): 5′-CGAGATTTTGGTCCCA-3′ and 3′-GGCACTTGTTGATAGA-5′. PCR products were separated by 2% agarose gel electrophoresis. PCR products subcloned into pcRII vector (Invitrogen) were sequenced on both strands by an automated DNA sequencer.

Quantitative RT-PCR was carried out using the one-step Taqman an EZ RT-PCR kit and Prism 7700 sequence detection system according to manufacturer’s protocols (PE Biosystems). 0.5-μg aliquots of RNA isolated from the above tissue samples were reverse transcribed and amplified using Tth DNA polymerase and the following primers and TaqMan probes: for ch-ERG, 5′-ACTCA GGCCGA GCTA TAAGC, 3′-GATCTCTTC-GCTTGCTCA, and TaqMan probe 5′-FAM-CTCCAGGTTAATTG-CATGCAGAA-TAMRA-3′ for C-1-1, 5′-CGCCAGATCTTCCCTGTCACA, 3′-TGATCTCTCCTGCTTGCTATA, and TaqMan probe 5′-FAM-CTACCTCAGAGAGAGGAGCCACTTT-TAMRA-3′. D ata were normalized to 18S ribosomal RNA simultaneously measured in each sample with the following primers: 5′-CGGTCA CCTA CACATCCA A G-GAA, 3′-GCTGGAATATCCGCGGCT, and TaqMan probe 5′-JOE-TGCTGGCA CACCAGTCCCTC-TAMRA-3′.

In Situ Hybridization

This procedure was carried out as described (Koyama et al., 1995) with some modifications. Tissue sections were pretreated with 1 μg/ml proteinase K for 1 min at room temperature and acetylated. Sections were hybridized with digoxigenin-labeled riboprobes synthesized according to manufacturer’s directions (Boehringer). Hybridization was performed as described (Koyama et al., 1995) with some modifications. Tissue sections were pretreated with 1 mg/ml proteinase K for 10 min at 37°C to unmask nuclear

Abbreviations used in this paper: A Pase, alkaline phosphatase; Ihh, Indian hedgehog; PTHrP, parathyroid hormone related protein; and RT-PCR, reverse transcriptase polymerase chain reaction.

The Journal of Cell Biology, Volume 150, 2000 28
antigens. Sections were then incubated for 2 h at room temperature with different dilutions of primary antibodies which were (a) rabbit affinity-purified antibodies to human ERG-1 (Santa Cruz Biotechnology; these antibodies cross-react with all variants of ERG and were originally raised against a peptide corresponding to COOH terminus amino acids 344–363 of human ERG-1 that is present in every ERG variant and is highly conserved among species); or (b) a rabbit antisemur specific for ch-ERG that we prepared. Peptide PLPHLTSDDYKALQNPSLM R corresponded to the region present in the ERG only was injected three times in different rabbits. Specificity of the antiserum used in this study (antisemur no. 46200) was verified by ELISA assays. A fter extensive rinsing, bound antibodies were revealed by the biot-in-avidin-peroxi-dase detection method, Histofine (Nichirei), or by the biotin-avidin-galac-tosidase method (Lim and Chae, 1989; Koyama et al., 1999). Slides were counterstained with fast green or un-stained and mounted and viewed by microscopy.

In experiments monitoring changes in tenasin-C distribution, concentration, and whether ch-ERG only was injected and whether C-1-1 only was injected, were isolated and processed for Northern blot analysis using chick tenasin-C and aggrecan cDNA clones as described previously (Pacifici et al., 1993).

Results

Cloning of C-1-1 and Expression Patterns of ch-ERG and C-1-1

In the first set of experiments, we asked whether ch-ERG is expressed at late stages of chick limb skeletogenesis and whether ch-ERG variants homologous to mammalian variants occur in chick and are expressed in cartilage. To this end, RNA was isolated from the cartilaginous femur or tibiotarsus of day 17 chick embryos and was processed for reverse transcription polymerase chain reaction (RT-PCR; Fig. 1 A). At this stage of development, the femur and tibiotarsus contain (a) a well-formed epiphysis and an overlaying articular chondrocyte layer and an overlaying thin fibrocartilage layer facing the synovial cavity, and (b) a long metaphyseal-diaphyseal region with growth plate chondrocytes undergoing maturation, hypertrophy, and endochondral ossification (Howlett, 1979; Pacifici, 1995). The PCR primers used were from sequences upstream and downstream of the 81- and 72-bp segments alternatively spliced in mammals (Fig. 1 B). We obtained two products of 473 and 392 bp (Fig. 1 A). Sequence analysis revealed that the 473-bp product encoded the previously described ch-ERG (Fig. 1 C), while the 392-bp product encoded an alternative and novel variant lacking the 81-bp segment only (Figs. 1 B and C). We named this avian variant C-1-1. No additional ch-ERG variants were obtained by 5′ and 3′ RACE analysis of cartilage samples (not shown).

To identify the chondrocyte population(s) expressing ch-ERG and/or C-1-1, longitudinal sections of day 17 chick embryo tibiotarsus were processed for in situ hybridization (Koyama et al., 1995). We used a common probe that hybridizes to both C-1-1 and ch-ERG, and a probe which is ch-ERG specific and encodes the 81-bp segment present only in ch-ERG. The common probe produced a strong hybridization signal in the articular chondrocyte layer and in the prehypertrophic zone of growth plate cartilage (Fig. 1 D, left panel). Signal was particularly strong in chondrocytes located along the border between the articular layer and overlaying fibrocartilage, but there was no signal in the fibrocartilage layer itself (Fig. 1 D, left panel, arrow-heads). In comparison, the ch-ERG-specific probe produced a strong signal only in the prehypertrophic zone (Fig. 1 D, right panel), suggesting that the signal in articular cartilage layer was largely due to C-1-1. Low signal was seen in the proliferative and hypertrophic zones with ei-
Biological Activities of ch-ERG and C-1-1 In Vivo

To determine the biological properties and possible functions of ch-ERG and C-1-1 in skeletogenesis, we ectopically expressed these factors in early chick limb buds using the replication-competent retroviral vector RCAS (Hughes et al., 1987) and determined the effects on long bone development at later stages. Accordingly, we injected concentrated ch-ERG or C-1-1 virus in the anterior mesenchymal region of the leg bud in stage 22–23 (day 3.5–4.0) chick embryos; alternatively, we implanted a small pellet of fibroblasts producing ch-ERG or C-1-1 viral particles in the same region. A control, companion embryos were implanted in the same position with insert-less RCAS virus or fibroblasts producing insert-less virus. Eggs were reincubated until day 10 (stage 36) of embryogenesis, and the effects of misexpression of ch-ERG or C-1-1 were determined by histology and in situ hybridization.

These procedures resulted in a high frequency of infection of the developing tibiotarsi. In control day 10 embryos, the tibiotarsi displayed characteristic histological features, including a proximal flat-shaped articulating epiphysis (Fig. 3 A, arrowhead), a distal round-shaped articulating epiphysis (Fig. 3 B), and a long metaphysal-diaphyseal shaft with growth plate chondrocytes undergoing maturation and ossification (Fig. 3 A, arrows). The diaphysis contained hypertrophic mineralizing cartilage, enchondral bone, and invading marrow (Fig. 3 E), and was surrounded by a well-formed intramembranous bone col-

| Tissue                | C-1-1 ± SD | ch-ERG ± SD | C-1-1/ch-ERG |
|-----------------------|------------|-------------|--------------|
| Articular cartilage   | 146 ± 33.0 | 52 ± 16.3   | 2.81         |
| Growth plate          | 35 ± 7.5   | 44 ± 4.2    | 0.80         |

was ~2.8 in articular cartilage and 0.8 in growth plate (Table 1).

To verify and extend these findings, we processed longitudinal sections of day 17–18 tibiotarsi for immunohistochemistry (Fig. 2), using a commercially available antiserum that reacts with every ERG variant (anti–ch-ERG/C-1-1) and an antiserum specific for ch-ERG that we prepared (anti–ch-ERG). The common ch-ERG/C-1-1 antiserum produced specific nuclear staining in both articular (Fig. 2 A) and prehypertrophic chondrocytes (Fig. 2, B and F); as also seen by in situ hybridization, expression was particularly strong in chondrocytes at the articular-fibrocartilage border (Fig. 2 A, arrowhead). This antiserum did not stain chondrocytes in the proliferative and hypertrophic zones (Fig. 2, E and G) and fibrocartilage cells (Fig. 2 A). In comparison, the ch-ERG–specific antibodies produced strong nuclear staining in prehypertrophic chondrocytes (Fig. 2 D) but much less staining in articular chondrocytes (Fig. 2 C). Other growth plate zones exhibited barely visible staining (not shown).

Taken together, the above data show that C-1-1 expression characterizes chondrocytes in the developing articular cartilage layer, while ch-ERG is preferentially produced by prehypertrophic chondrocytes in the growth plate.
lar (Fig. 3 E, arrowheads). When we examined ch-ERG virus-infected embryos, relatively few effects were seen. A slight longitudinal shortening of tibiotarsus was obtained in some cases (2/7). However, the overall histological organization of tibiotarsus was normal, and the diaphysis contained seemingly normal hypertrophic cartilage, endochondral and intramembranous bone (Fig. 3 G, arrowheads), and invading marrow. In sharp contrast, when we examined C-1-1 virus-infected embryos, we found that the tibiotarsus was significantly shorter and abnormally shaped compared with control (5/6) (Fig. 3, C and D). The epiphyses were severely abnormal; paradoxically, the proximal epiphysis was elongated (Fig. 3 C) and the distal one was broad and flatter (Fig. 3 D); that is, the reverse of control epiphyses. In addition, the metaphyseal-diaphyseal shaft displayed no hypertrophic cartilage (Fig. 3 C, arrow), no endochondral bone, no marrow, and no intramembranous bone collar (Fig. 3 F, arrowheads).

To further assess the effects of C-1-1 misexpression, longitudinal sections of day 10 control and C-1-1-infected legs were processed for in situ hybridization. In control tibiotarsus, expression of the cartilage characteristic type II collagen gene was strong throughout the cartilaginous tissues (Fig. 4 A) and type X collagen gene expression was restricted to hypertrophic chondrocytes (Fig. 4 B, arrows). In C-1-1-infected tibiotarsus, type II collagen expression was strong as in control (Fig. 4 D), but type X collagen gene expression was undetectable (Fig. 4 E), confirming that chondrocyte maturation has been halted. To correlate these gene expression patterns to viral distribution and C-1-1 misexpression, companion longitudinal sections...
were hybridized with radiolabeled ch-ERG/C-1-1 probe and exposed for a short and identical period of time so that hybridization signal levels were directly comparable. In control specimens, hybridization signal was relatively weak (given the short exposure; Fig. 4 C), whereas in C-1-1–infected specimens signal was extremely strong and present throughout the tibiotarsus and adjacent connective tissues (Fig. 4 F).

The severe phenotypic changes in the tibiotarsus of C-1-1–infected embryos were limited to the tibiotarsus itself, did not reflect systemic changes, and did not occur in neighboring uninfected skeletal elements. For example, the fibula next to the C-1-1–infected tibiotarsus shown in Fig. 3, C and D, above had remained uninfected and was morphologically and histologically normal (Fig. 5 A). It displayed abundant type II collagen transcripts in each region (Fig. 5 B) and abundant type X collagen transcripts in its hypertrophic diaphysis (Fig. 5 C), and contained low normal levels of ch-ERG/C-1-1 transcripts, whereas these transcripts were extremely abundant in the surrounding virus-infected perichondrial-mesenchymal tissues (Fig. 5 D).

Together, the results show that the biological activities of C-1-1 and ch-ERG are very different. C-1-1 appears to be a potent inhibitor of chondrocyte maturation and hypertrophy; this indicates that one function of this factor is to maintain chondrocytes in an immature and developmentally stable state, such as that displayed by articular chondrocytes. Instead, ch-ERG appears to have modest biological effectiveness.

**C-1-1, but Not ch-ERG, Induces Tenascin-C Gene Expression In Vivo and In Vitro**

Given the above conclusion, one would predict that C-1-1 should regulate and induce expression of phenotypic traits which are characteristic of articular and immature chondrocytes, while ch-ERG should not. Thus, we determined whether C-1-1 and ch-ERG differentially affect gene expression of tenascin-C, an extracellular matrix molecule produced by articular and immature chondrocytes but not produced by maturing growth plate chondrocytes (Pacifici et al., 1993; Savarese et al., 1996). In this respect, tenascin-C is a unique and rare phenotypic marker, given that articular and growth plate chondrocytes otherwise share numerous phenotypic traits, including collagens II, IX, and XI, link protein, aggrecan, hyaluronan, and others.

To test the above prediction in vivo, C-1-1 or ch-ERG virus was again injected in the leg bud of stage 22–23 chick embryos; contralateral leg bud was not injected and served as control. On day 10–11 of embryogenesis, legs were dissected, fixed, and embedded in paraffin; the resulting longitudinal serial sections were processed for immunohistochemistry, using the chick tenascin-C monoclonal antibody M1 (Chiquet and Fambrough, 1984). To compare the results directly, sections from infected and control legs were mounted on the same microscopic slides and simultaneously processed for immunohistochemistry. In this series of virus injections, both the tibiotarsus and metatarsal elements were infected. In control uninfected elements, tenascin-C displayed a characteristic distribution; it was confined to most epiphyseal developing articular chondrocytes (Fig. 6, A and C, arrows) and was largely absent from the long underlying growth plate (Pacifici et al., 1993). A similar distribution was seen in ch-ERG virus-infected elements (not shown). In sharp contrast, tenascin-C staining in C-1-1 virus-infected elements was both stronger and more widely distributed. In the abnormally shaped tibiotarsus, tenascin-C staining occupied a large portion of the epiphysis-metaphysis and was quite strong; it then decreased in the remainder of the shaft (Fig. 6 B). In the similarly abnormal metatarsal element, tenascin-C staining...
was actually detectable in each portion and was continuously present from proximal epiphysis to distal epiphysis (Fig. 6 D).

Tenascin-C can affect cell adhesion and morphology (Erickson, 1993). It is noteworthy that the wider tenascin-C distribution in C-1-1 virus-infected elements was accompanied by disturbances in the normal patterns of characteristic chondrocyte morphologies. For example, in control metatarsus the epiphyseal tenasin-C-positive incipient articular chondrocytes were round in shape (Fig. 6 E) and were followed by tenasin-C-negative proliferative growth plate chondrocytes exhibiting their typical flat and variously shaped morphology (Fig. 6 E; H owllet, 1979; Pacifici et al., 1993). In the C-1-1 virus-infected element, a clear transition from round to flat chondrocytes was absent; instead, most chondrocytes exhibited a roundish-to-oval morphology (Fig. 6 F) and most of them were tenasin-C rich (Fig. 6 D).

For an in vitro test of the hypothesis that C-1-1, but not ch-ERG, regulates expression of tenasin-C, we isolated resting chondrocytes from the caudal portion of day 17 chick embryo sternum, infected them with ch-ERG, C-1-1 or insert-less virus, and maintained them in culture for up to 5 wk with weekly subculturing (passages 1-5). Sternal chondrocytes were used because they can be isolated as homogenous populations, whereas chondrocyte populations isolated from limb skeletal elements usually contain contaminating perichondrial-fibroblastic cells (Gibson and Flint, 1985; Iwamoto et al., 1993a). We found that virally driven C-1-1 overexpression did lead to increased tenasin-C mRNA levels at each passage, but ch-ERG overexpression did not (Fig. 7 A). Gene expression of aggrecan, a chondrocyte characteristic matrix component, was not affected by any of the viruses (Fig. 7 A, A G); the slight variations seen with passage number were not significant and may reflect partial decrease in phenotypic expression over time in culture. In good agreement with the RNA analysis, immunocytochemistry on passage 2 cultures showed that tenasin-C was hardly detectable in control cultures but readily apparent in C-1-1-overexpressing cultures (Fig. 7 B). Previous studies showed that factors such as PTH/PTHrP, Indian hedgehog (Ihh) and fibroblast growth factor-2, act as negative modulators of maturation in growth plate chondrocytes (Iwamoto et al., 1995; Colvin et al., 1996; Vortkamp et al., 1996). Interestingly, neither treatment of sternal chondrocytes with any of these factors nor virally driven Ihh overexpression led to increased expression of tenasin-C (not shown).

To verify that the ch-ERG and C-1-1 viruses had caused the expected increases in ch-ERG and C-1-1 protein content, nuclear extracts were prepared from each of the above cultures and processed for Western blot analysis; we used the commercially available antiserum that reacts with every ERG variant (anti–ch-ERG/C-1-1), and the antiserum specific for ch-ERG that we prepared (anti–ch-ERG). We found that passage 1 chondrocytes infected with ch-ERG or C-1-1 virus already contained several fold more ch-ERG or C-1-1 protein than control cultures infected with insert-less virus (Fig. 7 C). Note that endogenous levels of ERG proteins in control cultures, though difficult to document in Fig. 7 C, were appreciable by visual inspection and became readily apparent after longer development of the blots (not shown).

Together, the above data show that C-1-1 can selectively induce expression of tenasin-C, a unique product of articular and immature chondrocytes, but ch-ERG can not. In addition, C-1-1 misexpression and the concurrent increases in tenasin-C distribution are accompanied by disturbances in chondrocyte cytoarchitecture.

Opposite Effects of ch-ERG and C-1-1 on Alkaline Phosphatase and Mineralization

In a final set of experiments, we further compared the biological properties of ch-ERG and C-1-1. We reasoned that if C-1-1 maintains chondrocytes in an immature state, it should inhibit alkaline phosphatase activity that is strongly expressed by mature hypertrophic chondrocytes (Leboy et al., 1989); however, ch-ERG should have no effect. To test this prediction, chondrocytes were isolated from the cephalic maturing portion of day 17 chick embryo sterna and grown in culture up to passage 4 by weekly subculturing. A Pase activity was monitored at each passage. We found that, as expected, A Pase activity had increased about threefold in control cultures between passage 2 and 3, a reflection of the ongoing and advancing maturation process (Fig. 8 A). In line with our hypothesis, we found that such increase had also occurred in ch-ERG but not in C-1-1-infected cultures (Fig. 8 A). In older passage 4 cultures, we again observed that A Pase activity had increased further in control and that no detectable A Pase was seen in C-1-1 cultures. Unexpectedly, however, A Pase activity in ch-
ERG cultures now exceeded that in control by nearly two-fold (Fig. 8 A).

Because APase activity is associated with the mineralization phase of the maturation process (Ali et al., 1970), we asked whether the very large increase in APase activity in late passage ch-ERG cultures may be associated with mineral deposition in the extracellular matrix. Confluent passage 4–5 cultures of control, ch-ERG, and C-1-1 chondrocytes in microwell plates were grown for four additional days in medium containing 25 μg/ml ascorbate, an additive needed for mineralization in culture (Leboy et al., 1989). Cultures (in duplicate) were then processed for histochemical detection of mineral, using alizarin red or von Kossa stains. Indeed, we found that while some mineral was present in control cultures, much larger amounts of it were present in ch-ERG cultures (Fig. 8 B). In agreement with the antimaturation ability of C-1-1 demonstrated above, we found that mineral was completely absent in C-1-1 cultures (Fig. 8 B).

**Discussion**

In this study, we have demonstrated that developing chick limb skeletal elements express two variants of the transcription factor ERG, the full-length ch-ERG previously described by Dhordain et al. (1995) and a novel shorter variant (C-1-1) lacking a 27-amino acid segment located ~80 amino acid upstream of the DNA-binding ETS domain. We demonstrate that the gene expression patterns and biological activities of ch-ERG and C-1-1 are distinct. C-1-1 characterizes developing epiphyseal articular chondrocytes, whereas ch-ERG is preferentially expressed by prehypertrophic chondrocytes in the growth plate and to a lesser extent by articular chondrocytes. When constitutively expressed, C-1-1 maintains chondrocytes in an immature and phenotypically stable phenotype, blocks their progression into type X collagen- and APase-rich hypertrophic chondrocytes, and induces expression of tenasin-C, a unique trait of articular and immature chondrocytes. In addition, C-1-1 blocks endochondral ossification and marrow invasion and the formation of an intramembranous bone collar. In comparison, when constitutively expressed in vitro, ch-ERG promotes chondrocyte development and expression of functions associated with the terminal phases of maturation, namely APase activity and mineral deposition in the extracellular matrix. The data indicate that C-1-1 and ch-ERG participate, and have distinct roles, in limb skeletogenesis. Because of its expression patterns and its powerful and distinct biological activity, C-1-1 may be instrumental in the genesis of articular chondrocytes and in the acquisition and maintenance by these cells of their most important feature, a stable and immature phenotype. On the other hand, given its ability to promote chondrocyte maturation in vitro and its weak activity in vivo, ch-ERG may have auxiliary roles in skeletogenesis and may be one among several factors that facilitate completion of chondrocyte maturation and transition from mineralized hypertrophic cartilage to endochondral bone.
Development of Articular and Growth Plate Chondrocytes

As pointed out in the introduction, while the understanding of articular chondrocyte development is quite limited at present, there is a large body of information on the development of growth plate chondrocytes. Thus, it has become apparent that the progression of growth plate chondrocytes through the resting, proliferative, prehypertrophic, hypertrophic, and mineralizing phases of maturation is regulated by a number of powerful factors and molecules, including insulin-like growth factors, fibroblast growth factors, bone morphogenetic proteins, hedgehog proteins, and retinoids (Colvin et al., 1996; Vortkamp et al., 1996; Enomoto-Iwamoto et al., 1998; Koyama et al., 1999). It is of interest that these various factors and molecules have contrasting effects on chondrocyte maturation. For instance, fibroblast growth factors and their tyrosine kinase receptors are found to exert a negative role on both chondrocyte proliferation and progression toward the hypertrophic stage (Colvin et al., 1996; Naski et al., 1998; Sahni et al., 1999). On the other hand, we have shown that endogenous retinoids and their nuclear receptors play a positive role on maturation and are required for chondrocyte hypertrophy, mineralization, and endochondral ossification (Koyama et al., 1999). Together, these studies have clearly indicated that the maturation process of growth plate chondrocytes is regulated by a fine balance between negative and positive mechanisms; that is, mechanisms that oppose maturation and mechanisms that favor it.

It follows that the development of articular chondrocytes may require a shift in such balance in favor of negative mechanisms, so that the maturation process would be effectively and irreversibly blocked and the cells would maintain a stable and immature phenotype through life (Pacifici et al., 1990). C-1-1 and ch-ERG may represent one example of such a shift. We show that these factors are expressed in both the articular layer and growth plate, but at very different ratios. In the articular layer the C-1-1/ch-ERG expression ratio is ~2.8, whereas in the growth plate it is ~0.8. Thus, by being more strongly expressed in the articular layer, C-1-1 may be able to exert an effective antimaturation action, maintain the cells in an immature stage, and participate in the regulation of traits characteristic of that stage; these traits include a small cell size, low...
A Pase activity, lack of type X collagen synthesis, lack of matrix mineral deposition, and strong tenascin-C synthesis. On the other hand, a shift in the C-1-1/ch-ERG expression ratio to ~0.8 in the growth plate, combined with preferential expression of these factors at such a ratio in the prehypertrophic zone (see Figs. 1 D and 2 F), may produce a change in biological activity, with ch-ERG becoming preponderant and exerting a promaturation effect.

An important cytological characteristic of developing articular chondrocytes is that the cells are round in shape, whereas the underlying proliferative growth plate chondrocytes are flat and variously shaped (Howlett, 1979; Lutfi, 1974; Shimazu et al., 1996). In addition, articular chondrocytes are surrounded by abundant amounts of tenascin-C, whereas the growth plate chondrocytes are tenascin-C negative (Pacifici et al., 1993; Savarese et al., 1996). We have shown here that C-1-1 misexpression induces broader and higher expression of tenascin-C; this is accompanied by alterations in chondrocyte morphology, with most cells exhibiting a roundish-to-oval morphology. Thus, C-1-1 appears to be able to directly or indirectly induce two features, i.e., a round cell shape and tenascin-C synthesis, which normally distinguish articular chondrocytes from underlying growth plate chondrocytes and which may be critical for the genesis of articular chondrocytes themselves. In other cell types, tenascin-C plays an antiadhesive role and cells plated onto tenascin-C-coated substrates remain round (Erickson, 1993). The protein could have a similar role in articular chondrocytes; if so, induction of tenascin-C and a round cell configuration would be causally linked and dependent on C-1-1 action.

An additional point deserves to be made with regard to tenascin-C induction by C-1-1. Other known negative modulators of the maturation process we tested, including Ihh, PTHrP and FGF-2, failed to induce tenascin-C gene expression in cultured chondrocytes. This finding is actually not surprising in view of the fact that Ihh and PTHrP, but not tenascin-C, are normally expressed in the growth plate (Vortkamp et al., 1996). Thus, our finding suggests an important implication: the antimaturation mechanisms such as those due to C-1-1, which allow developing epiphyseal articular chondrocytes to acquire and maintain a stable immature phenotype, may be different from those serving as negative modulators of maturation rates in growth plate chondrocytes.

**Ets Factors and Limb Development**

Our results complement well and extend previous work on the roles of ets factors in limb skeletogenesis. In situ hybridization studies in chick showed that ERG transcripts are first present in the prechondrogenic mesenchymal cell condensations in the early limb (Dohrdain et al., 1995) and subsequently at sites of future joint development (Gan et al., 1996; Macias et al., 1997). Related studies showed that in mouse embryo limbs another member of the ets family, ETS-2, is expressed throughout the mesenchyme and then becomes restricted to differentiated chondrocytes (M Aroulakou et al., 1994), and that ETS-2 misexpression in transgenic mice leads to severe skeletal defects (Sumarsono et al., 1996). It was not established in these studies whether the ERG expressed in the mesenchymal condensations is ch-ERG, C-1-1 or another variant, whether ERG or its variants are expressed by interzone cells, which constitute the primordial joint and give rise to several joint structures (Mitrovic, 1978), and whether ETS-2 is expressed by every chondrocyte present in long bone anlagen or is expressed only by specific populations. Nonetheless, it is clear from these studies and our data that at least two members of the ets family, ETS-2 and ERG, participate in limb skeletogenesis and that their functions are likely to be important and exerted at multiple stages of the process. Thus, the early, partially overlapping expression of ERG and ETS-2 in limb mesenchyme suggests that these factors may first cooperate in the cytodifferentiation process of condensed mesenchymal cells into chondrocytes. The subsequent, more distinct patterns of expression indicate that ERG and ETS-2 may start acting more independently, with ERG having a role in the onset of joint formation and ETS-2 operating in differentiated chondrocytes. Finally, our results indicate that ERG variants may ultimately play regionally specialized roles within each skeletal anlage.

Ets factors regulate transcription by forming heterodimeric complexes with family members or other transcription factors (Wasylyk et al., 1993, 1998; Graves, 1998). ETS-2 and ERG strongly interact with each other and can individually interact with c-Fos/c-Jun, producing heterotypic complexes with diverse biological activities and specificities (Butticé et al., 1996; Basuyaux et al., 1997). Interestingly, c-Fos, c-Jun, and other A P-1 complex members are expressed by both immature and hypertrophic chick chondrocytes, with c-Jun expression levels higher in immature than mature cells; in addition, virally driven misexpression of c-Fos or c-Jun in cultured chondrocytes and in the limb inhibits maturation and long bone development (Kameda et al., 1997; Watanabe et al., 1997), precisely as C-1-1 misexpression does. Thus, it is plausible that the biological functions of C-1-1 and ch-ERG in skeletogenesis involve interactions with ETS-2 and c-Fos/c-Jun. In particular, given the similar phenotypic consequences of their misexpression, C-1-1 and c-Fos/c-Jun may interact to maintain chondrocytes in an immature state and induce expression of traits characteristic of that state, such as tenascin-C. In preliminary studies (to be reported elsewhere), we have found that virally driven C-1-1 expression induces strong activity of a tenascin-C gene promoter-reporter construct, indicating that C-1-1 acts on the promoter directly. We have also found that the tenascin-C promoter contains a small element around position -300 that includes an ets consensus site adjacent to an A P-1 site; this element is reminiscent of biologically active ERG/AP-1-responsive element previously identified in other genes, such as that in the mammalian collagenase 1 gene (Butticé et al., 1996). Thus, such an element may similarly regulate tenascin-C gene expression in articular chondrocytes via interaction with C-1-1/c-Fos/c-Jun complexes.

**Structural Basis of ERG Function**

Work in mammalian systems has shown that ERG is most closely related to certain members of the ets gene family, including ETS-1, ETS-2, FLI 1, and G A B P (Wasylyk et al., 1997, 1998). All these factors are found to contain do
mains upstream and downstream of the ETS DNA-binding domain (see schematic in Fig. 9A) that modulate the factors' transcription activation abilities. The ETS domain, termed domain E, is located in the carboxyl half of the protein and contains a winged helix-loop-helix responsible for DNA binding. Domains A and C are transcriptional activator domains, domain B contains a helix-loop-helix thought to mediate protein–protein interactions (Seth and Papas, 1990), and domains D and F are regulators of DNA binding. For example, deletion of domain F reduces the ability of human ERG-1 and ERG-2 to activate ets motif-containing reporter constructs by >65% (Siddique et al., 1993).

ch-ERG shares >95% identity in amino acid sequence with its human counterpart ERG-3 (Dhordain et al., 1995) and thus, the structural organization of ch-ERG is undoubtedly quite similar to that of mammalian ERG as well as that of the other ets members above. What could then be the possible structural reasons for the significantly different biological properties of ch-ERG and C-1-1 we demonstrate here? The 27-amino acid segment present in ch-ERG and absent in C-1-1 represents the amino half of domain C (Fig. 9A). Preliminary computer-based models of the secondary structure of domains B, C, and D in ch-ERG and C-1-1 are shown in Fig. 9B. Both domains B and D are predicted to have a globular structure, whereas domain C has an extended configuration. In ch-ERG, domain C protrudes away from domains B and D and displays two close turns. Instead, domain C in C-1-1 (indicated as C1 in Fig. 9B) is obviously much smaller and contains a readily apparent antiparallel β-sheet with a single turn. In addition, the overall spatial arrangement of domains B, C, and D seems to be different in C-1-1 and ch-ERG; the domains appear to be closer to, and more aligned with, each other in C-1-1 than ch-ERG. Obviously, these models await experimental validation. Nonetheless, they suggest that the predicted significant differences in domain C could have short- and long-range repercussions on the proteins, possibly altering protein-protein and protein-DNA properties and leading to the diverse biological properties of ch-ERG and C-1-1. Domains of ets factors, including domain C, have been shown to be targets of phosphorylation (Rabault and Ghysdael, 1994; Wasylyk et al., 1997). If domain C in ERG turns out to be a similar target and if the presence or absence of the 27-amino acid segment affects phosphorylation, this pathway could also contribute to the diverse biological properties of ch-ERG and C-1-1.

We should mention that previous studies on human ERG-1, ERG-2 and ERG-3 found no major functional differences among these alternatively spliced variants (Duterque-Coquillaud et al., 1993; Prasad et al., 1994). The proteins exhibited similar DNA-binding affinity and similar ability to transactivate ets motif-containing reporter constructs. These observations would appear to disagree with our findings, but they probably do not. First, since the mammalian counterpart of C-1-1 has not yet been cloned, it was not included in the comparative analysis of ERG-1, ERG-2, and ERG-3. Second, it is possible that ERG-1, ERG-2, and ERG-3 may actually possess distinct biological properties, which, however, are not revealed by DNA binding and transactivation of reporter constructs. Biological assays more faithful to the in vivo situation may be required to reveal functional differences among variants, such as those used in our study or in studies of other ets variants (Melet et al., 1996).

In conclusion, the data in our study provide evidence that C-1-1 may have an important role in the genesis and function of articular chondrocytes. As far as we know, this
is the first transcription factor that, by virtue of its expression patterns and distinct biological activity, has been linked to the development of these cells. Ongoing work should provide details on how C-1-1 exerts this important cellular role and in turn, contributes to the formation and life-long function of diarthroidal joints.

We thank Dr. E. Ellis Golub for help with ProtPlot protein modeling program, Ms. E. Leann Golden for help with immunostaining, and Drs. S. A. Dams, P. Billings, P. Leboy, and I. Shapiro for comments and suggestions. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan to M. Iwamoto, and National Institutes of Health grant R01 A R 4600-01 to M. Pacifici and J. Rosenthal.

Submitted: 10 January 2000
R revised: 1 May 2000
A accepted: 19 May 2000

References

A. L., S.W. Saidera, and H. C. Ander. 1970. Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. Proc. Natl. Acad. Sci. USA, 67:1525–1529.

A. Rutter, C.W., H. Morgan, and A. A. Pitsillides. 1994. Cellular aspects of the development of diarthroidal joints and articular cartilage. J. Anat. 184:447–456.

B. Ayasuya, J. P., E. Feinhausen, D. Stehelin, and G. Buttì. 1997. The etS transcription factors interact with each other and with the Fos/Zip-1 complex under distinct protein domains in a DNAb-dependent and -independent manner. J. Biol. Chem. 272:2618–2619.

G. Buttì, G., M. Duterque-Coquillaud, J. P. Basayn, S. Carrere, M. Kurkin, and D. Stehelin. 1996. Ets family member, differentially regulates collagenase (1 M MP1) and stromelysin (MP 73) expression by physically interacting with the Fos/jun complex. Oncogene. 13:2307–2300.

C. Chiquet, M., and D. M. Fambrough. 1984. Chick myostatin antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. J. Cell Biol. 98:1926–1936.

C. Colms, J. S., B. A. Bohn, G. W. Harding, D. M. Esen, and D. M. Ornitz. 1996. Skeletal overgrowth and deathness in mice lacking fibroblast growth factor receptor. J. Nature Genet. 12:390–397.

D. Dordoin, P., F. Dewitte, X. Diensthein, D. Stehelin, and M. Duterque-Coquillaud. 1995. Mesodermal expression of the chicken ets gene associated with precartilaginous condensation and cartilage differentiation. Mech. Dev. 50:17–28.

D. Duterque-Coquillaud, M., C. Niel, S. Plaza, and D. Stehelin. 1993. New human ets isoforms generated by alternative splicing are transcriptional activators. Oncogene. 8:1963–1973.

E. Nomioto-Iwamoto, M., M. Iwamoto, Y. Musukui, Y. Kawakami, T. Nozuno, Y. Higuchi, S. Takemoto, H. Ohuchi, S. Noji, and K. Kurisu. 1998. Bone morphogenetic protein signaling is required for maintenance of differentiated phenotype, control of proliferation, and hypertrophy in chondrocytes. J. Cell Biol. 140:409–418.

E. Ricker, H. P. 1993. Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions. Curr. Opin. Cell Biol. 5:869–876.

Ganana, Y., D. Macias, M. Duterque-Coquillaud, M. A. Ros, and J. M. Hurle. 1996. Role of TGF beta and BMP-2 and BMP-7 in programmed cell death and skeletogenesis during limb development. Development. 124:1109–1117.

G. Arakaluk, I. C., T. S. Papas, and J. E. Green. 1994. Differential expression of et-s-1 and et-s-2 proto-oncogenes during murine embryogenesis. Oncogene. 9:1551–1565.

M. Melet, F., B. Motro, D. J. Ross, L. Zhang, and A. Bernstein. 1996. Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythrolymphakemia. Mol. Cell. Biol. 16:2708–2718.

C. Mitrovic, D. 1998. Development of the diarthroidal joints in the rat embryo. An. M. 125:1475–1485.

C. Mitrovic, D. 1997. Development of the metatarsalpalangeal joint in the chick embryo: Morphological, ultrastructural and histochemical studies. An. M. 150:333–348.

C. Naski, M. C., J. C. Colvin, J. D. Coffin, and D. M. Ornitz. 1998. Regress of hepatic signaling and BMP 4 expression in growth plate cartilage by fibroblast growth factor receptor 3. Development. 275:497–4988.

M. Pacifici. 1995. Tenascin-C and the development of articular cartilage. Matrix Biol. 14:689–698.

M. Pacifici, M. E., B. Golden, O. Oshima, I. M. Shapiro, P. S. Leboy, and S. L. A. Dams. 1990. Hypertrophic chondrocytes. The terminal stage of differentiation in the chondrogenic cell lineage? In Cell Lineages in Development. Vol. 599. F. A. Pepe, D. E. Boettiger, M. Pacifici, N. A., Rabinstein, and J. W. Sanger, editors. New York: A cademy of Sciences. New York. 45–57.

M. Pacifici, M., M. Iwamoto, E. B. Golden, J. L. Leatherman, Y. S. Lee, and C. M. Chuong. 1993. Tenascin is associated with articular cartilage development. Dev. Dyn. 198:123–134.

P. Prasad, D., V. N. Rao, L. Lee, and E. S. Reddy. 1994. Differentially spliced et3-3 product functions as a transcriptional activator. Oncogene. 9:69–73.

B. Rabau, B., L. D. Ghyssael. 1994. Calcium-induced phosphorylation of ETS 1 inhibits its specific DNA binding activity. J. Biol. Chem. 269:143–151.

T. S. Papas, and E. S. Reddy. 1987. Er et, a human ets-related gene on chromosome 21: alternative splicing, polyadenylation, and translation. Science. 237:635–639.

D. Savarese, J. H., E. Ricker, and P. S. Scully. 1996. A ricular chondrocyte tenascin-C production and assembly into de novo extracellular matrix. J. Orthop. Res. 14:273–281.

T. Selet, M., E. S. Reddy, and T. S. Papas. 1990. The c-ets-1 proto-oncogene has oncogenic activity and is positively autoregulated. Oncogene. 5:1761–1767.

M. Shimaza, A., H. D. N. Tah, H. Kuris, E. Koyama, J. L. Leatherman, E. B. Golden, R. A. Kosher, and M. Pacifici. 1996. Syndecan-3 and the control of chondrocyte proliferation during endochondral ossification. Exp. Cell Res. 229:126–136.

C. Siddle, H. R., V. N. Rao, I. S. Lee, and E. S. Reddy. 1993. Characterization of the DNA binding and transcriptional activation domains of the erg protein. Oncogene. 8:1751–1755.

D. Sumarsono, S. H., T. J. Wilson, M. J. Tymms, D. J. O'Venter, M. C. Corrick, R. Kola, M. H. Lahoud, T. S. Papas, A. Seth, and I. Kola. 1996. Down’s syndrome-like skeletal abnormalities in Ets2 transgenic mice. Nature. 379:534–537.

V. Vortkamp, A., K. C. Lane, G. V. Segre, H. M. Kronenberg, and C. J. Wern.
Tabin, 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science. 273:613-622.
Wasylyk, B., and A. Nordheim. 1997. Ets transcription factors: partners in the integration of signal responses. In Transcription Factors in Eukaryotes. Vol. 1. A.G. Papavassiliou, editor. Chapman & Hall, New York. 253-286.
Wasylyk, B., S.L. Hahn, and A. Giovane. 1993. The Ets family of transcription factors. Eur. J. Biochem. 211:7-18.
Wasylyk, C., A.P. Bradford, A. Gutierrez-Hartmann, and B. Wasylyk. 1997. Conserved mechanisms of Ras regulation of evolutionary related transcription factors, Ets 1 and Pointed P2. Oncogene. 14:899-913.
Wasylyk, B., J. Hageman, and A. Gutierrez-Hartmann. 1998. Ets transcription factors: nuclear effectors of the Ras-MAPK kinase signaling pathway. Trends Biochem. Sciences. 23:213-216.
Watanabe, H., K. Saitoh, T. Kameda, M. Murakami, Y. Niikura, S. Okazaki, Y. Morishita, S. Mori, Y. Yokouchi, A. Kuroiwa, and H. Iba. 1997. Chondrocytes as a specific target of ectopic Fos expression in early development. Proc. Natl. Acad. Sci. USA. 94:3994-3999.