INTRODUCTION
Fractures are healed when new bone formation bridges the fractured site. Two mechanisms are responsible for this new bone formation, intramembranous bone formation which occurs adjacent to the fracture site, and endochondral bone formation which occurs at the fracture gap. We previously showed that fracture healing is delayed with increasing animal age. During fracture repair in six-month-old rats, cartilage formation and subsequent endochondral ossification were profoundly decreased when compared to one-month-old animals, while intramembranous bone formation was relatively unchanged (1).

 Sox9, a member of Sox (Sry-type HMG box) gene family, encodes a transcription factor required for type II collagen gene expression in chondrocytes, and expression of Sox9 has been reported in embryonic cartilage and in the cartilaginous portion of the fracture callus (1-4). Other members of the Sox gene family, Sox6 and L-Sox5, function in cooperation with Sox9 as activators of type II collagen expression (4), and these genes are coexpressed in developing embryonic cartilage tissues.

We have initiated studies to identify mechanisms underlying cartilage formation in the callus that are affected by age. In this study we evaluate expression of Sox genes (Sox9, Sox6, and L-Sox5) in fractures made in the femur of one and six month old rats.

MATERIAL & METHODS
Fracture model: Fractures were made in male one- and six-month-old Long-Evans rats by the method of Bonnarens and Einhorn (5). Rats were sacrificed 2, 4, 7, 10, or 15 days after fracture. Animal care was conducted in accordance with the "Principals of Laboratory Animal Care" formulated by the National Institutes for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health (NIH Publication No 86-23, revised 1985).

RNA extraction from fracture callus: Fracture calluses were collected from 10 animals at each time point, snap frozen in liquid N2, and pulverized. Pulverized calluses from each group were mixed. RNA was extracted from 10 animals at each time point, snap frozen in liquid N2, and pulverized.

Chondrocyte cell culture and RNA extraction: Chondrocytes were isolated from the distal femoral epiphyses of neonatal rat by digestion with 1 mg/ml collagenase and 0.25% trypsin. Isolated cells were cultured in DMEM containing 10% FBS and antibiotics. Total RNA was extracted from confluent cell cultures by the method of Chomczynski et al. (6).

Relative quantification of Sox genes and type II collagen gene expression by RT-PCR: Gene expression was quantified on the basis of PCR kinetics (7). First-strand cDNA libraries were synthesized from total RNA using random primer and AMV-RT (reverse transcriptase). PCR primers for Sox genes, type II collagen and GAPDH (an internal control) were designed using the coding sequences of human, rat, or mouse genes. PCR amplification was performed with [a-32P]-dCTP over a wide range of PCR cycles. Incorporation of [a-32P]-dCTP into the desired amplified product was measured by Phosphorimager (Molecular Dynamics) after electrophoresis. The PCR product yield curve (cycle #vs. PCR product) was plotted for each reaction mixture, and the initial amount of template was estimated using the exponentially increasing region of each curve. The relative values for Sox genes and type II collagen were obtained by comparison with the internal standard, GAPDH.

RESULTS RT-PCR for Sox9, Sox6, type II collagen, or GAPDH using cultured rat chondrocyte RNA showed a single major band of the expected length for each PCR product. Direct sequencing of each PCR product showed a high homology with human Sox9 or mouse Sox6 sequences, and identity with rat type II collagen and rat GAPDH sequences. RT-PCR for Sox5 using rat chondrocyte RNA resulted two major bands. Direct sequencing of these bands showed homology with the mouse L-Sox5 and Sox5L sequences.

Relative quantification of mRNA coding for type II procollagen and for Sox genes in rat fracture callus was determined in one- and six-month-old rats.

DISCUSSION We evaluated expression of Sox genes (Sox9, Sox6, L-Sox5 and Sox5L) in cultured rat chondrocytes and rat fracture callus. Expression of these genes in the fracture callus from adult rats implies that in addition to activating type II procollagen expression in the embryo, Sox genes also function to regulate chondrogenesis in adult tissues. In our previous work (1) we showed immunoreactivity against Sox9 in immature chondrocytes in the fracture but not in mature chondrocytes. The time course of Sox genes expression during fracture healing process and differences in gene expression between one- and six-month-old rats also suggest that Sox genes may be more involved in chondrocyte differentiation than in maturation.

We found expression of the Sox5L gene in cultured rat chondrocytes and fracture callus. Unlike Sox 9, Sox6, and L-Sox5, the functional significance of Sox5L expression has not been elucidated.

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REFERENCE