Functional Hierarchy between Two OSE2 Elements in the Control of Osteocalcin Gene Expression in Vivo*

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Osteocalcin gene expression is initiated perinatally and is restricted to mature osteoblasts and odontoblasts. Because their pattern of expression is highly restricted, the osteocalcin genes are excellent tools to study osteoblast-specific gene expression. To define the mechanisms of osteocalcin cell-specific gene expression in vivo, we generated transgenic mice harboring deletion mutants of the promoter region of OG2, one of the mouse osteocalcin genes. We show here that only 647 base pairs of this promoter are sufficient to confer cell-specific and time-specific expression to a reporter gene in vivo. This fragment promoter contains two copies of OSE2. This osteoblast-specific cis-acting element binds Osf2, a recently characterized osteoblast-specific transcription factor (Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) Cell 89, 747–754). We also demonstrate that the proximal OSE2 element is critical to confer an osteoblast-specific, developmentally regulated pattern of expression to a reporter gene. The other OSE2 element, located more upstream and presenting a lower affinity for Osf2, affects only weakly OG2 promoter activity. These data demonstrate the crucial role of Osf2 in controlling osteocalcin gene expression. Since osteocalcin synthesis is a hallmark of the differentiated osteoblast phenotype, these results suggest that, beyond its developmental function, Osf2 is also required for the maintenance of the osteoblast phenotype postnatally.

The osteoblast, or bone-forming cell, is a cell type of mesenchymal origin that is responsible for bone matrix deposition (2, 3). A defect, relative or absolute, in osteoblast function such as is observed in various skeletal dysplasias or in osteoporosis has major consequences on bone architecture and remodeling and may even be lethal under some conditions (4–6). This underlines the importance of understanding the molecular mechanisms controlling osteoblast-specific gene expression and thereby maintenance of the phenotype of this cell type.

Major progress has been made recently in our understanding of the mechanisms controlling osteoblast-specific gene expression using both the type I collagen and osteocalcin genes. The osteocalcin genes are excellent tools to address this question since they are expressed in vivo in osteoblasts and odontoblasts, their tooth counterparts, and in no other extracellular matrix-producing cells (7, 8). In mouse, there are two osteocalcin-encoding genes expressed in osteoblasts and odontoblasts only, OG1 and OG2 (9). They encode the same protein and display the same expression pattern, and their proximal promoter regions are 93% identical over the first 1.3 kb1 (9, 10). To understand the mechanisms leading to osteocalcin cell-specific gene expression, we and others have studied the promoter element of these genes. In tissue culture experiments, a 200-bp fragment of the rat promoter (11) and a 160-bp fragment of the mouse OG2 promoter (10) have been shown to be necessary and sufficient to confer osteoblast-specific expression to a reporter gene. We characterized two osteoblast-specific cis-acting elements in this OG2 promoter fragment (termed OSE1 and OSE2) that were able to confer osteoblast-specific activity to a heterologous promoter (10). We subsequently focused our attention on OSE2 and Osf2, the factor binding to it. We recently cloned Osf2/Cbfa1, hereafter called Osf2, the transcript of the Cbfa1 gene encoding the nuclear activity binding to OSE2 (1). Osf2 is expressed only in cells of the osteoblastic lineage during development; after birth, it regulates the expression of several genes only or predominantly expressed in osteoblasts, and forced expression of Osf2 in non-osteoblastic cells leads to osteoblast-specific gene expression (1). Moreover, genetic evidence in mice and humans showed that total absence or haploinsufficiency of Osf2 leads to an arrest of or a defect in osteoblast differentiation (12–15). These data demonstrated that this factor is critical for osteoblast differentiation and that its function is non-redundant with other factors during development.

In view of these recent advances in our understanding of osteoblast differentiation, it was important to analyze the mechanisms controlling the osteoblast-specific expression of the osteocalcin genes in vivo. In this study, we narrowed down the region necessary for osteoblast-specific expression to a 647-bp fragment of the murine OG2 promoter and showed that this fragment contains two OSE2 elements. Analysis of their respective binding abilities showed that the most proximal element has greater affinity for Osf2 than the distal one. Furthermore, site-specific mutagenesis experiments demonstrated the existence of a functional hierarchy between these two OSE2 elements. Tissue culture experiments as well as studies of various transgenic mouse strains revealed that the most proximal site accounts for most of the promoter activity, whereas

1 The abbreviations used are: kb, kilobase(s); bp, base pair(s); X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; EMSA, electrophoretic mobility shift assay.
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**Fig. 1.** 1316 bp of the OG2 promoter confer bone-specific expression in transgenic mice. A, schematic representation of the p1316-luc transgene; B, luciferase (Luc) activities in tissue extracts of 4-week-old mice from two independent expressing lines. Luciferase activities are expressed as light units/100 μg of protein. Values are means of data from three to five transgenic animals.

The second site contributes only weakly to OG2 promoter activity. These data demonstrate the critical role of Osf2 in regulating osteocalcin gene expression, suggesting that Osf2 may control the maintenance of the osteoblast phenotype.

**EXPERIMENTAL PROCEDURES**

DNA Constructions—Construction of the p1316-luc and p647-luc plasmids has been previously described (10). p647-lacZ was generated by subcloning the −647/+13 OG2 promoter fragment into pLacF (16). Mutations were introduced in the OSE2 sites, termed OSE2a and OSE2b, by means of the two-step polymerase chain reaction method (17). Primers used for OSE2a mutagenesis were 5’-CTTATAGAACCCAAGACCATGGC-3’ (−674/−652), 5’-TGCTGTTCTTGGTGATTGCAGC-3’ (−149/−128), and 5’-TGATGGTGACTTGTCTGT-3’ (+13/+3). Primers used for OSE2b mutagenesis were 5’-TTTATAGAACCCAAGACCATGGC-3’ (−674/−652), 5’-CTTCTCCCCCACAAGAACAAGA-3’ (−620/−598), 5’-TTCTTTCTTTGGGGGAAAGAG-3’ (−598/−620), and 5’-TGATGGTGACTTGTCTGT-3’ (+13/+3). Sequences of the polymerase chain reaction products were verified by automatic DNA sequencing. Mutated promoter fragments were cloned in the pLuc promoterless luciferase expression vector (18).

Generation of Transgenic Mice—Plasmids were digested with appropriate restriction enzymes, and the insert was purified by two rounds of agarose gel electrophoresis. Linear DNA inserts were injected into the pronuclei of fertilized B6D2F1 mouse eggs (Charles River Laboratories, Inc.). In the case of the 647-bp promoter fragment, luciferase and lacZ constructs were coinjected to obtain transgenic mice coexpressing the two transgenes. Histochemical staining for lacZ expression provides precise details about the cell type expressing the transgene in the context of the entire animal, whereas luciferase expression is measured by a very sensitive assay that allows a quantitative determination of the level of expression. Animals expressing the transgenes were identified by screening for luciferase activity in tails. The copy number of integrated sequence was determined in F1 generation animals by Southern blot analysis according to Brinster et al. (19) with the following modifications. 10 μg of tail genomic DNA was digested by EcoRI and BglII and fractionated by gel electrophoresis in the presence of calibrated amounts of control DNA (luciferase- and lacZ-linearized plasmids) for a range of 1–32 copies per haploid genome. After transfer to Hybond-N membranes (Amersham Pharmacia Biotech), the DNA was hybridized with the 0.6-kb HindIII/EcoRI fragment of the luciferase gene or the 1-kb NcoI/ClaI fragment of the lacZ gene. Transgene copy number was estimated by comparison of the intensity of the hybridization signal with the DNA control lanes.

Analysis of Transgenic Mice—Luciferase expression in tissues was assessed in 4-week-old F1 animals. Organs were dissected and homogenized on ice in buffer containing 100 mM potassium phosphate (pH 7.8) and 1 mM dithiothreitol. Protein homogenates were centrifuged, and supernatants were assayed for luciferase activity according to standard protocols (10). Protein levels were measured using the Bio-Rad protein assay. Relative luciferase activities were expressed as luciferase light units/100 μg of protein.

LacZ activity was analyzed in transgenic mouse lines by staining skinned and eviscerated 2- and 4-day-old pups with X-gal using conventional techniques (20). After staining, specimens were partially cleared in 70% ethanol for several weeks. X-gal staining of primary osteoblast cultures was performed as described (20). RNA of primary osteoblasts in culture was extracted with RNAzol (Cinna Biotech) following the manufacturer’s instructions. Northern blotting was performed according to standard procedures (17) using the mouse OG2 cDNA as a probe (9).

Nuclear Extracts and DNA Binding Assays—Nuclear extracts from ROS 17/2.8 cells were prepared by the method of Dignam et al. (21) using buffers containing 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and pepstatin. DNase I footprint assays were performed on the NcoI/BglII and BglII/SalI fragments of the −647/+13 OG2 promoter fragment, labeled asymmetrically.
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647 bp of the OG2 Promoter Are Sufficient to Direct Bone-specific Expression in Transgenic Mice—To delineate the minimum OG2 promoter fragment required for osteoblast-specific expression of a reporter gene in vivo, we created transgenic mice containing two different OG2 promoter fragments fused to the lacZ reporter gene and/or the luciferase reporter gene (Figs. 1A and 2A). For each construct, at least two founder animals were generated and analyzed. Southern blot analysis indicated that all lines harbored comparable copy numbers of the transgene (four to eight copies).

We first analyzed the expression of the 1316-bp luciferase chimeric gene in calvariae, long bones, and a number of soft tissues. As shown in Fig. 1B, the level of luciferase activity was high in calvariae and long bones and was not significant in any of the soft tissues examined, indicating that the 1316-bp promoter fragment contains cis-acting elements required for tissue-specific expression.

We next tried to define a shorter OG2 promoter fragment able to confer high level osteoblast-specific expression to a reporter gene in vivo. When we used a 647-bp OG2 promoter fragment to drive luciferase expression, we observed that this transgene was also highly expressed in bone (Fig. 2B). Again, no significant luciferase activity was detected in any soft tissues examined, except for the brain in one line (Fig. 2B). Ectopic expression of the reporter gene in brain has been previously reported in transgenic mice harboring a 3.9-kb human osteocalcin promoter fragment (24). This could be explained by the absence of a brain-specific cis-acting repressor element in...
the promoter fragments used. Alternatively, this expression could reflect the activity of a cryptic regulatory element present in the reporter cassettes used to generate the transgene. Upon insertion in a particular genomic context, this element would become active and confer expression in brain. Results presented below suggest that this latter hypothesis is correct. Regardless of the brain expression in one line, these results indicate that 647 bp of the \( O^{2}G \) promoter contain all the regulatory elements necessary and sufficient to direct bone expression of a reporter gene.

The 647-bp \( O^{2}G \) Promoter Fragment Confers Proper Cellular and Temporal Expression to a Reporter Gene—In the case of the 647-bp promoter fragment, each transgenic line was generated by coinjection of the luciferase and \( \text{lacZ} \) constructs (Fig. 2A). To assess the cell-specific activity of the 647-bp \( O^{2}G \) promoter fragment, 2-day-old F1 pups were analyzed by whole-mount X-gal staining. As shown in Fig. 3A, every bone of the skeleton expressed the transgene and was stained in blue. In the appendicular skeleton, high levels of LacZ activity were present in the diaphyses of the long bones, but not in the cartilaginous regions of the joints (Fig. 3B). Similarly, in the axial skeleton, the ossified vertebral bodies and ribs proper stained in blue, whereas the central marrow tissues, the cartilaginous intervertebral discs, and the costal cartilages were not stained (Fig. 3, A and C). In the head, all the bones of the skull were stained. At postnatal day 2, lighter staining was noted at the edges of the sutures, where ossification is still not complete at this stage of development (Fig. 3D, left). As expected, this difference in staining was greatly reduced when older animals (4 days old) were examined (Fig. 3D, right). No soft tissues showed \( \text{lacZ} \) expression in any of the lines examined. In particular, cross-sections through the head of the animals from all the lines, including the one with a high level of luciferase activity in brain, failed to detect any X-gal staining in brain (Fig. 3E). This result suggests that the luciferase expression in brain, in this particular line, may result at least partly from the activity of a cryptic cis-acting element present in the luciferase reporter cassette.

To compare the temporal expression of the \( \text{lacZ} \) gene in these mice with endogenous osteocalcin gene expression, we used a well characterized model of primary osteoblast culture (25). This technique allows isolation of osteoblast progenitors that can differentiate and eventually form mineralized bone nodules when placed in ascorbic acid- and \( \beta \)-glycerophosphate-supplemented medium. Osteocalcin gene expression can be observed in these cultures only when the osteoblasts become fully differentiated (25, 26). Primary osteoblast cultures derived from calvariae of transgenic animals were established and assayed...
expression of the osteocalcin genes. As shown in Fig. 4 (A and B), no expression of the transgene or of endogenous osteocalcin genes was detected in immature cultures (0 days of culture). In contrast, X-gal staining and endogenous osteocalcin gene expression were observed in bone nodules (10 and 15 days of culture). These results indicate that the 647-bp OG2 promoter fragment contains the cis-acting elements required for cell-specific and time-specific expression of the osteocalcin genes in vivo.

Two OSE2 Elements Are Present in the First 647 bp of the OG2 Promoter—Given its strict cell-specific activity, we next focused our attention on the 647-bp fragment. DNA sequence analysis revealed the presence of a bona fide OSE2 element (located between −608 and −602) and of an OSE2-like element (located between −526 and −520) (Fig. 5A). Identical sites are present at the same location in the OG1 promoter, the other mouse osteocalcin gene (data not shown). The sequence of the −608/−602 site was identical to the originally described OSE2 element located between −137 and −131 (Fig. 5A). The −526/−520 site presented a single base pair mutation that has been shown to abolish Osf2 binding (1, 10). We termed the originally described OSE2 as OSE2a and the −608/−602 OSE2 as OSE2b. Because of the single base pair mutation, the sequence located between −526 and −520 was termed OSE2-like. No OSE1 element other than that previously described (10) was found in the 647-bp OG2 promoter fragment. Likewise, we did not detect any sequence similar to the osteoblast-specific cis-acting element present in the Col1a1 promoter (18, 27, 28). The location of the osteoblast-specific cis-acting elements present in the 647-bp promoter fragment is indicated in a schematic representation of the OG2 promoter (Fig. 5A). DNase I footprint analysis of the −647−147 region using nuclear extracts from mouse primary osteoblasts and from ROS 17/2.8 osteoblastic cells showed the existence of a protected region between −614 and −596 that includes OSE2b (Fig. 5B). More important, no protection was observed at the location of the OSE2-like site when using primary osteoblast nuclear extracts. This is in agreement with the inability of recombinant Osf2 to bind to that sequence (1). No other osteoblast-specific binding sites were observed (data not shown).

EMSAs were performed to establish that OSE2b was a bona fide OSE2 element. We first used nuclear extracts to assess the affinity of Osf2 for OSE2b. When we used in EMSA a wild-type OSE2a oligonucleotide as a probe and ROS 17/2.8 nuclear extracts as a source of proteins, a specific protein-DNA complex was observed that was competed away by the wild-type OSE2a oligonucleotide, but not by a mutant OSE2a oligonucleotide (Fig. 6A, lanes 1–7). This complex was competed away by a 2-fold higher molar excess of wild-type OSE2b oligonucleotide (Fig. 6A, lanes 8–13), whereas a mutant OSE2b oligonucleotide could not abolish binding of nuclear factors to this oligonucleotide (lane 14). We then used the OSE2b oligonucleotide as a probe in EMSA and unlabeled OSE2a and OSE2b oligonucleotides as competitors. The complex formed between ROS 17/2.8 nuclear extracts and OSE2b had the same electrophoretic characteristics as the one observed when using the OSE2a oligonucleotide as a probe (Fig. 6, compare A, lane 1, and B, lane 1). This complex was better competed by the wild-type OSE2a oligonucleotide than by OSE2b itself (Fig. 6B, lanes 2–5 and 8–11). Again, none of the mutant oligonucleotides were able to compete for the binding of nuclear factors to the labeled OSE2a oligonucleotide (Fig. 6B, lanes 6 and 12). These experiments indicate that the affinity of the Osf2 activity present in ROS 17/2.8 nuclear extracts was higher for OSE2a than for OSE2b. Consistent with these findings, recombinant Osf2 (1) bound to OSE2a and OSE2b; however, five times more recombinant protein was needed to see binding to OSE2b compared with OSE2a (Fig. 6C, lanes 1 and 3). Taken together, these results demonstrate that Osf2 has a different affinity for OSE2a and OSE2b. Since both elements harbor the same core binding sequence, this difference in binding ability suggests that surrounding sequences can modulate the affinity of Osf2 for the DNA.

OSE2a Is the Main Regulator of the Activity of the 647-bp OG2 Promoter Fragment in Tissue Culture—The DNA binding data presented above raised the possibility that these two OSE2 elements may have different functions. To test this hypothesis, we compared the activity of the wild-type 647-bp promoter fragment with that of the same fragment in which either one of the two OSE2 elements was mutated. Transient DNA transfection experiments were performed in two different osteoblastic cell lines, rat ROS 17/2.8 and mouse MC3T3-C4 cells (23). Identical results were obtained for each cell line. Mutation of OSE2a led to a 80% decrease in the promoter activity, whereas the same mutation in OSE2b led to a 30–40% decrease (Fig. 7, A and B). When both OSE2 elements were mutated, the activity of the promoter was almost identical to the activity of the mutant promoter containing a mutation only in OSE2a (Fig. 7, A and B). We hypothesize that the residual activity observed in the absence of both OSE2 elements is conferred on the promoter by OSE1, the other osteoblast-spe-
cific cis-acting element present in the OG2 promoter (10). These results, consistent with our previous deletion analysis in DNA transfection (10), demonstrate that, in cell culture, OSE2a is the most efficient cis-acting element and that, in its absence, OSE2b alone cannot maintain the level of activity of the promoter.

FIG. 6. OSE2b binds Osf2 with a lower affinity than OSE2a. DNA binding was analyzed by EMSA. A, labeled wild-type OSE2a oligonucleotide was incubated with ROS 17/2.8 nuclear extracts (lanes 1–14). Competition experiments were performed with the indicated molar excess of unlabeled wild-type OSE2a oligonucleotide (lanes 2–6), mutant OSE2a oligonucleotide (OSE2am; lane 7), wild-type OSE2b oligonucleotide (lanes 9–13), or mutant OSE2b oligonucleotide (OSE2bm; lane 14). B, labeled wild-type OSE2b oligonucleotide was incubated with ROS 17/2.8 nuclear extracts (lanes 1–12). Competition experiments were performed with the indicated molar excess of unlabeled wild-type OSE2b oligonucleotide (lanes 2–5), mutant OSE2b oligonucleotide (lane 6), wild-type OSE2a oligonucleotide (lanes 8–11), or mutant OSE2a oligonucleotide (lane 12). C, DNA binding was performed with His-Osf2. Labeled probes were as indicated.

FIG. 7. Functional hierarchy between OSE2a and OSE2b in cell culture experiments. Shown are the results from the functional analysis of p647-luc constructs harboring a mutation in either or both OSE2a and OSE2b elements. Respective activities of these constructs were analyzed by DNA transfection experiments in ROS 17/2.8 rat cells (A) and in MC3T3-C4 mouse cells (B). Schematic representations of the reporter constructs are shown on the left. Mutated sites are indicated by gray boxes. Values are percentages of the wild-type promoter activity. Data represent ratios of luciferase (Luc)/β-galactosidase activities (A) or luciferase/chloramphenicol acetyltransferase activities (B), and values are means of six to eight independent transfection experiments; error bars represent S.D. OSE2am, mutant OSE2a oligonucleotide; OSE2bm, mutant OSE2b oligonucleotide.

OSE2a Is Required for OG2 Promoter Activity in Vivo—We next used an in vivo approach to analyze the respective importance of the two OSE2 sites. Transgenic mice containing the 647-bp promoter fragment with either one of the two OSE2 elements mutated fused to the luciferase reporter gene were generated (Fig. 8A). F1 animals from independent expressing
A p647-luc gene fusions

FIG. 8. OSE2a is a major regulator of the 647-bp OG2 promoter fragment in vivo. Transgenic mice lines were generated that contain mutated versions of the 647-bp OG2 promoter fused to the luciferase reporter gene. Mutated sites are indicated by gray boxes. A, luciferase (Luc) activities measured in tissues of 4-week-old F1 animals. B, effect of a mutation in the OSE2b element. C, effect of a mutation in the OSE2a element. Luciferase activities are expressed as light units/100 μg of proteins. Values are means of data from three to five transgenic animals. OSE2am, mutant OSE2a oligonucleotide; OSE2bm, mutant OSE2b oligonucleotide.

Discussion

This study was designed to define the mechanisms regulating osteocalcin gene expression in vivo. Our results demonstrate that 647 bp of the OG2 promoter are necessary and sufficient to confer osteoblast-specific expression to a reporter gene in vivo. To our knowledge, this is the first demonstration that osteoblast-specific expression can be achieved using an osteocalcin promoter fragment shorter than 1.7 kb (29). This short promoter fragment now provides a useful tool to express various reporter genes of interest specifically in osteoblasts.

Ectopic expression of the transgene in brain was observed in one line using the luciferase marker gene, but not the lacZ reporter gene. This discrepancy probably reflects the potential of all reporter genes to contain sequences that can contribute to transgene expression (30). This emphasizes the need to use different reporter constructs in parallel to accurately assess transgene expression in transgenic mice. Beside its ability to confer osteoblast-specific expression, the 647-bp OG2 promoter can also recapitulate the appropriate temporal expression of osteocalcin genes. Driving the lacZ reporter gene, this short promoter fragment confers not only an osteoblast-restricted expression, but also a time-specific expression. As is the case for the osteocalcin genes, the transgene is not expressed in newborn calvaria-derived osteoblast cultures, but becomes active after 10 days in the condensing cells of the mineralization nodules. This result, as well as the critical role played by the OSE2a site in controlling OG2 promoter activity, indicates that this promoter presents a simple organization. Its activity is mainly regulated positively, through osteoblast-specific cis-acting elements. This result is not surprising considering the exquisite cell-specific expression of this gene.

Sequence analysis of the 647-bp region revealed the existence of two potential OSE2 sites located 383 and 465 bp, respectively, upstream of the previously described proximal site, OSE2a (10). DNase I footprint assays demonstrated that only the most distal site present in the mouse promoter (OSE2b, –608/—602) bound nuclear proteins. No novel or previously described (10, 18, 26, 27) osteoblast-specific cis-acting elements were detected. Three OSE2-like elements have also been reported in the rat osteocalcin promoter (31). However, no functional studies are available to assess the respective role of each of these sites.

The presence of two OSE2 elements in the mouse osteocalcin
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promoter raised the question as to their respective function in vivo. A site-directed mutagenesis analysis of the two OSE2 sites revealed that OSE2a was functionally more important than OSE2b. This is consistent with the higher affinity of Osf2 for OSE2a. Indeed, OSE2a is a major regulator of OSE2 promoter activity if not the only active OSE2 element. More important, OSE2b was not able to compensate for the absence of OSE2a. These results demonstrate that the presence of consensus OSE2 sites, as well as their ability to bind Osf2 in EMSA, is not sufficient to define their function and underscores the importance of performing functional studies. Our results also raise the question of the OSE1 function and suggest that this element may have only an accessory role in controlling osteoblast-specific gene expression. Alternatively, OSE1 may act cooperatively with OSE2. These issues will become testable only when the protein binding to OSE1 becomes available.

We have previously shown that 160 bp of the OPG2 promoter can direct osteoblast-specific expression in tissue culture experiments (10). When we tested this short promoter fragment in transgenic mice, we observed a very low but specific expression in bone in two out of eight lines (data not shown). This discrepancy between the tissue culture and in vivo experiments (10) arises from an OSE2a fragment of interest. Although OSE1 is a major regulator of osteocalcin gene expression, our study suggests that it is required for OSE2a activity if not the only active OSE2 element. More importantly, Osf2 could play a dual role in osteoblasts. First, during embryogenesis, it would trigger the differentiation process along the osteoblast lineage. Second, during life, Osf2 would control osteoblast function, maintaining the expression of specific gene products required for bone matrix deposition. This hypothesis can now be tested directly in vivo.

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