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Analysis of the pattern of *QM* expression during mouse development

Abstract *QM*, a novel gene that was originally identified as a putative tumor suppressor gene, has since been cloned from species encompassing members of the plant, animal, and fungal kingdoms. Sequence comparison indicates that *QM* has been highly conserved throughout eukaryotic evolution. *QM* is a member of a multigene family in both mouse and man, is expressed in a broad range of tissues, and is downregulated during adipocyte differentiation. *Jif*-1, a chicken homolog of *QM*, has been reported to interact with the protooncogene *c-Jun*, and to inhibit transactivation of AP-1 regulated promoters in vitro. Furthermore, disruption of the yeast *QM* homolog is lethal. Although these studies suggest that the *QM* gene product plays an important role within the normal cell, the precise role of *QM* has remained elusive. In this study, a thorough analysis of the pattern of *QM* expression during mouse development was undertaken, using the techniques of whole mount in situ hybridization and whole mount immunohistochemistry, in combination with conventional immunohistochemical analysis of tissue sections. *QM* is expressed in numerous embryonic tissues, and is differentially expressed throughout the embryo. The cytoplasmic localization of *QM* is consistent with its reported association with ribosomes, and inconsistent with its previously hypothesized function as a direct modulator of the nuclear protooncogene *c-Jun*. *QM* is expressed in the developing epidermis, and is particularly strong within developing limbs.

Analysis of embryos of various stages of gestation indicate that *QM* is downregulated in the surface ectoderm of the embryo as development proceeds. *QM* protein is not detectable within either nucleated or enucleated red blood cell precursors. *QM* is strongly expressed within chondrocytes within the transition zone of developing limb cartilage, as well as within differentiated keratinocytes of the suprabasal regions of the epidermis. Furthermore, within both cartilage and skin, there is an inverse relationship between *QM* expression and proliferative capacity. This pattern of *QM* expression suggests that this novel gene product may be involved in processes such as posttranslational protein processing which are essential for differentiation of specific tissues during embryogenesis.

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Introduction

Human *QM* cDNA was originally cloned by subtractive hybridization between a tumorigenic Wilms' tumor cell line (G401), and a nontumorigenic microcell hybrid (G401.6) during a search for a Wilms' tumor suppressor gene [2]. Human *QM* cDNA encodes a hydrophilic, basic, cytosolic protein with a predicted molecular weight of 25 kDa [2, 8, 19]. The *QM* protein was completely novel at the time of its cloning, lacking any functional motifs indicative of protein function. Various *QM* cDNAs have since been cloned from diverse species, encompassing members of the plant, animal, and fungal kingdoms [6]. Sequence comparison of various *QM* cDNAs indicates that the *QM* protein has been highly conserved throughout eukaryotic evolution. For example, murine and human *QM* differ by only a single amino acid [6]. This high degree of conservation is indicative of a gene whose product provides an essential function in vivo.

The role of *QM* is presently unknown; however, several studies suggest that the *QM* protein plays an important role in the normal cell. The chicken homolog of *QM*, *Jif*-1, was reported to interact with the protooncogene *c-Jun* and to inhibit transactivation of AP-1 regulated promoters in vitro [14]. However, studies in several experimental systems indicate that *QM* is a cytoplasmic protein,

and subcellular fractionation studies in both yeast and man indicate that *QM* localizes to the rough endoplasmic reticulum and is ribosome associated [5, 12]. Recent studies suggest that the proposed interaction between *QM*/*Jif*-1 and *c-Jun* in regulation of the activation of AP-1-containing promoters could be an experimental artifact [11]. In other studies, *QM* has been reported to be downregulated during adipocyte differentiation [4]. Regardless of the role(s) of the *QM* gene product, *QM* provides an essential function in yeast, since deletion of *QM* is lethal [9, 18]. Furthermore, human *QM* cDNA can function to rescue this lethality (B. Trumpower, personal communication), indicating that the high degree of evolutionary conservation observed between *QM* homologs obtained from species as diverse as yeast and man is functionally significant.

Previous expression studies have indicated that *QM* transcript is detectable in many mammalian tissues and cell lines [2, 8]; however, a detailed study of the pattern of *QM* expression is lacking. In this study, we have analyzed the pattern of *QM* expression within the developing mouse throughout embryogenesis. The technique of whole mount in situ hybridization using a riboprobe specific for murine *QM* was used to analyze the presence of *QM* transcript. We have also analyzed the pattern of *QM* protein expression using the techniques of whole mount immunohistochemistry and section immunohistochemistry using an antibody raised against a C-terminal peptide of *QM*. In addition, the relationship between *QM* expression and cellular proliferation was investigated. These analyses have uncovered patterns of *QM* expression that have been informative in defining the function of the *QM* gene product in vivo.

Methods

Harvesting of embryonic tissue

Embryos harvested from Swiss-Webster-timed pregnant mice (Simonsen Laboratories) were fixed according to their intended use. Embryos to be used for whole mount in situ hybridization were fixed overnight at room temperature in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), transferred to 100% methanol, and stored at –20ºC. Embryonic tissue to be used for whole mount immunohistochemistry was fixed in 4% paraformaldehyde for 2–4 h at 4°C and stored in 100% methanol at –20°C. When embryonic tissue was to be used for section immunohistochemistry, the embryos were fixed in 4% paraformaldehyde for 2–4 h at 4°C and stored in 70% ethanol at –20°C.

Generation of riboprobes

The plasmid construct used as a template for the generation of the *QM* riboprobe consisted of a 443 nucleotide PCR-generated fragment that contained the last four exons of murine *QM* cDNA cloned into pBluescript SK (Stratagene). Digoxigenin-labeled riboprobes were synthesized by in vitro transcription of the linearized DNA template using digoxigenin UTP (Boehringer Mannheim) according to the manufacturer's recommendation. The probe was diluted to a concentration of 10 μg/ml in hybridization buffer [50% formamide, 5X SSC (20X SSC: 3 M NaCl and 0.3 M NaCitrate, pH 7.2), 1 mg/ml yeast RNA, 100 μg/ml heparin, 1X Denhardt's solution, 0.1% tween-20, 0.1% CHAPS, 5 mM EDTA], and was stored at –20°C until use.

Whole mount hybridization

Whole mount in situ hybridization was performed based on procedures described by Wilkinson and Green [22]. Briefly, embryos were rehydrated in a methanol series, washed in PTw (1X PBS + 0.1% tween-20), and treated with proteinase K (Boehringer Mannheim) in PTw at 37°C. The concentration of proteinase K and incubation time were optimized for each embryonic stage in order to allow for probe penetration without sacrificing tissue morphology, and ranged from 2.5 μg/ml for 5 min (E 9.5 embryos), to 20 μg/ml for 20 min (E 12.5 embryos). Embryos were acetylated in 0.1 M triethanolamine containing 0.5% acetic anhydride, fixed in 3.7% formaldehyde, washed several times in PTw, and prehybridized in hybridization solution at 60°C for 5 h. The digoxigenin-labeled riboprobe was denatured, diluted in hybridization solution to a final concentration of 1 μg/ml, and hybridization was performed overnight at 60°C. Posthybridization washes were performed at 65°C in hybridization solution for 10 min, three times in 2X SSC for 20 min each, and twice in 0.2X SSC for 30 min each. Immunohistochemistry washes were as follows: twice in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 15 min at room temperature, 1 h in MAB + 2 mg/ml BSA, and 6 h at 4°C in blocking solution (MAB + 2 mg/ml BSA + 20% heat-inactivated sheep serum). Antidigoxigenin IgG-alkaline phosphatase (Boehringer Mannheim) was preabsorbed in blocking solution overnight at 4°C, and the supernatant was diluted 1:1000 in blocking solution and incubated with the embryos overnight at 4ºC. Unbound antibody was removed by extensive washing in MAB for 30 min each and twice in AP reaction buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 15 min each. Colorimetric detection was performed by incubation with 0.5 ml BM purple substrate (Boehringer Mannheim) for 1 h, and embryos were fixed in phosphate-buffered formalin (2 g $NaH_2PO_4 \cdot H_2O$, 3.3 g Na_2HPO_4, 50 ml 37% formaldehyde per 500 ml) for 15 min, rinsed in 1X PBS, and stored at 4°C in 1X PBS + 0.002% sodium azide. Embryos were photographed using a Wild Epimacroscope M450 (McBain Instruments) equipped with surface illumination.

Whole mount immunohistochemistry

Embryos were incubated in 5:1 methanol:30% hydrogen peroxide for 3–5 h and transferred to 100% methanol overnight at –20°C. Embryos were rehydrated in sequential incubations in 50% methanol, 15% methanol, and 1X PBS for 30 minutes each at 4°C, followed by incubation twice in PBSMT (2% nonfat dry milk, 0.1% Triton X-100 in 1X PBS) for 1 h each. *QM* C-17 polyclonal antibody (Santa Cruz Biotechnology, 100 μg/0.1 ml) was diluted 1:500 in PBSMT and incubated with embryos overnight at 4°C. Unbound primary antibody was removed by washing twice in PBSMT at 4°C for 1 h each, and three times in PBSMT for 1 h each at room temperature. Goat antirabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, 200 μg/0.5 ml) was diluted 1:300 to 1:900 in PBSMT. The tissues were incubated with diluted secondary antibody overnight at 4°C.

Unbound secondary antibody was removed by extensive washing as for the primary antibody, above. Embryos were washed with PBT (1X PBS, 0.1% Triton X-100) for 20 min and preincubated with horseradish peroxidase substrate (0.3 mg/ml 3', 3' diaminobenzidine, 0.5% $NiCl_2$, in PBT) for 30 min. The colorimetric reaction was initiated by the addition of 0.003% hydrogen peroxide, the reaction was incubated for a period of 15 min to 1 h, and the embryos were rinsed in PBT and stored at 4°C in 1X PBS + 0.002% sodium azide. Embryos were photographed as previously mentioned for the technique of whole mount in situ hybridization.

Sectioning of embryonic tissue

Embryos were dehydrated in an ethanol series ending in 100% ethanol. Dehydrated tissues were incubated in a 1:1 mixture of 100% ethanol:PEW [Polyester wax: 90% poly (ethylene glycol-400) disteatrate (Aldrich) and 10% 1-hexadecanol (Aldrich)] at 40°C under vacuum

Fig. 1A, B Whole mount immunohistochemistry: E 8.5 (embryonic day 8.5) and E 9.5 embryos. E 8.5 embryos **(A)** and E 9.5 embryos **(B)** were immunohistochemically stained using a *QM* polyclonal antibody. *QM* protein was detected as a purple colorimetric product

for 15 min, and embedded in fresh PEW. Seven-micron sections were cut as ribbons using a Spencer 820 Microtome (Fritz Instruments) and mounted on Superfrost Plus microscope slides (Fisher).

Immunohistochemistry of embryo sections

Slides were washed in 100% ethanol to remove the PEW, airdried briefly, and incubated in 9:1 methanol:30% hydrogen peroxide for 10–30 min. Tissues were rehydrated in PBS and incubated for 30 min in blocking solution (1 mg/ml BSA/1X PBS). *QM* C-17 polyclonal antibody (100 µg/0.1 ml, Santa Cruz Biotechnology) was diluted 1:50 in blocking solution and was incubated with the tissue overnight. Unbound antibody was removed by washing in PBS, and goat antirabbit IgG-horseradish peroxidase (200 µg/0.5 ml; Santa Cruz Biotechnology) diluted 1:50 in blocking solution was applied to sections and incubated for 1 h. Unbound secondary antibody was removed by washing as in the previous steps, and tissues were incubated with horseradish peroxidase substrate (0.3 mg/ml 3', 3', diaminobenzidine, 50 mM Tris, pH 7.5, 0.0003% hydrogen peroxide) for 5–10 min. Sections were washed, dehydrated in an ethanol series ending in 100% ethanol, rinsed in xy-

lene, and mounted in Histomount (Zymed). Tissues were photographed using phase contrast and light condensers on a Olympus BH-2 microscope (McBain Instruments).

In vivo cellular proliferation assay

Animals were injected intraperitoneally with 1 ml BrdU labeling reagent (Zymed) per 100 g of body weight for a period of 1 h, the animals were sacrificed, and embryos were harvested and pro-

Fig. 2A–D Whole mount in situ hybridization: murine *QM* expression during development. E 10 embryos **(A)**, E 11 embryos **(B)**, E 12 embryos **(C)**, and E 13 embryos **(D)** were probed with an antisense murine *QM* riboprobe. *QM* transcript was detected as a blue colorimetric product
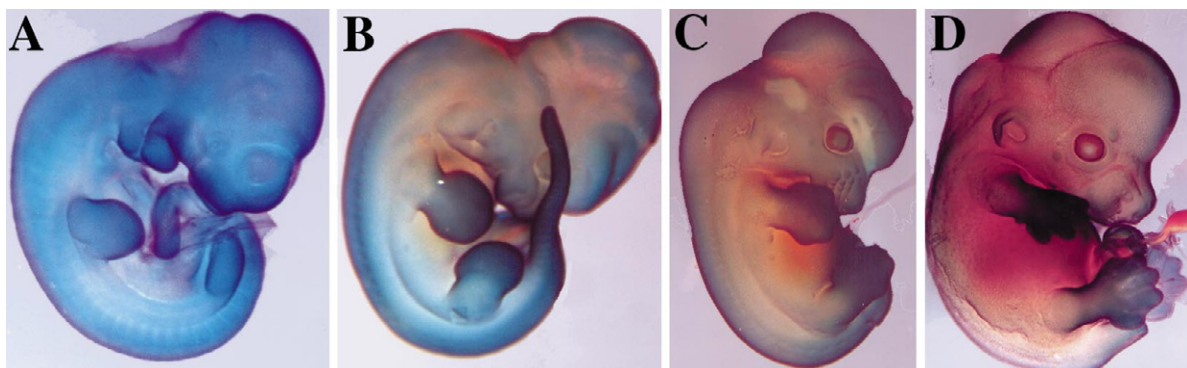
cessed for PEW embedding as described above. Tissues were embedded in polyester wax, sectioned, and immunohistochemically stained using an in vivo cell proliferation assay kit (Zymed) according to the manufacturer's protocol.

Results

Analysis of the pattern of *QM* protein during murine development by whole mountimmunohistochemistry

Wild-type embryos were subjected to whole mount immunohistochemistry using an antibody that specifically recognizes the *QM* protein. Figure 1 shows an E 8.5 (embryonic day 8.5) embryo (Fig. 1A) and an E 9.5 embryo (Fig. 1B) that has been subjected to whole mount immunohistochemistry using the anti-*QM* antibody. *QM* protein is detected in a very broad pattern on the surface of the embryo at both stages of development. The expression of *QM* at the protein level is more restricted as development proceeds, as is evident by comparing *QM* expression in the E 9.5 embryo to that of the E 8.5 embryo. This downregulation of the *QM* protein as development progresses parallels the pattern observed for that of *QM* transcript (see Fig. 2).

Fig. 3A–D Whole mount immunohistochemistry: sections of embryos that had been analyzed for *QM* protein. E 10.5 embryos that had been immunohistochemically stained for *QM* protein were sagittally sectioned and analyzed microscopically. A section through the developing limb **(A)** is shown at higher magnification **(B)** *and* indicates that the *QM* protein is very highly expressed within the developing epidermis. A section through the developing brain **(C)** is shown at higher magnification **(D)** and indicates that the pattern of *QM* protein is cytoplasmic (**A, C** ×100, **B, D** ×400, *Ep* developing epidermis, *M* mesenchyme, *E* eye, *V* ventricle of the brain, *O* optic stalk)
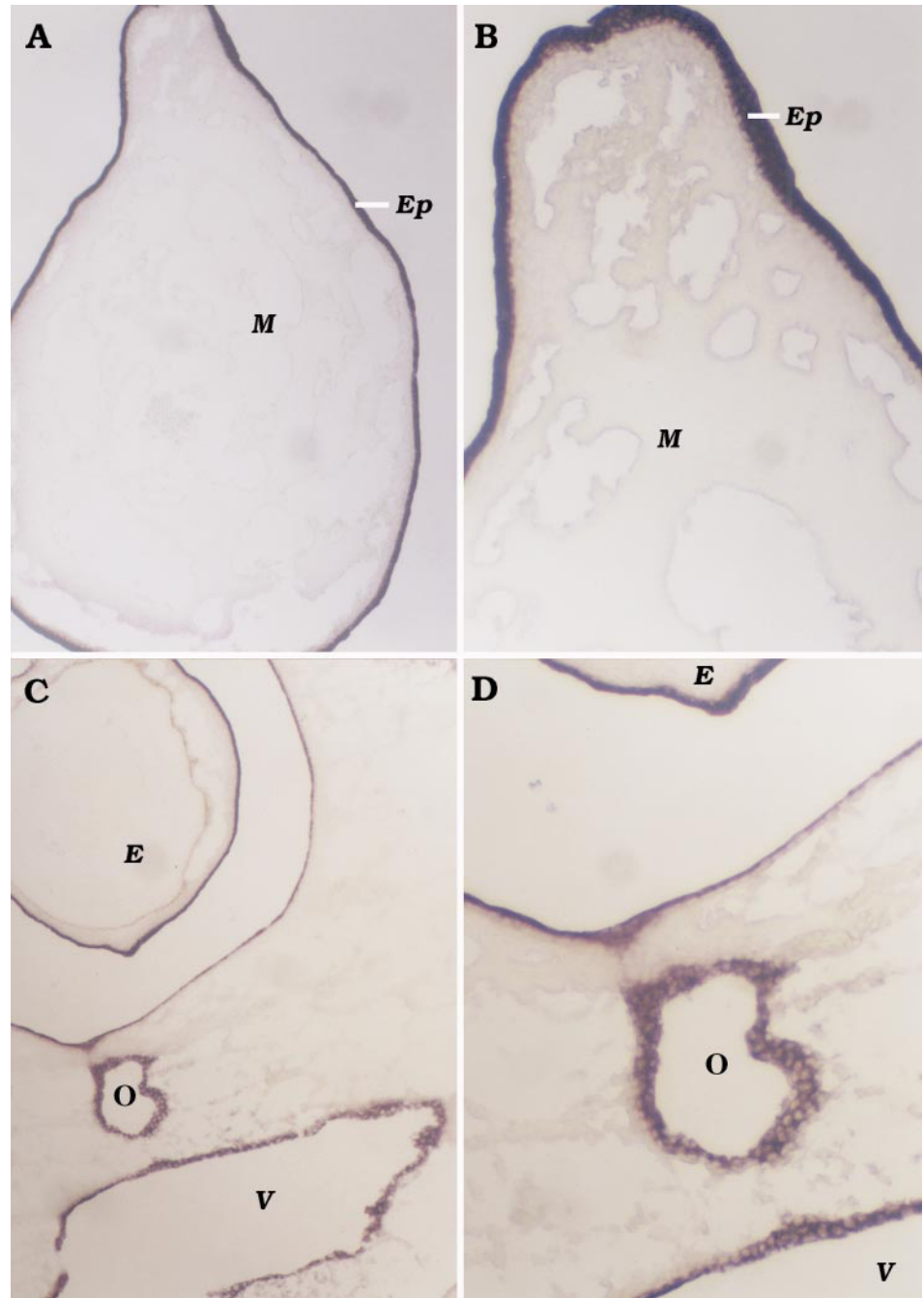
Analysis of the pattern of *QM* transcript during murine development by whole mount in situ hybridization

Midgestation embryos were were analyzed for the pattern of *QM* expression. Embryos were staged by observance of a vaginal plug estimated to be 0.5 days post coitum (E 0.5). Embryos at days E 10, E 11, E 12, and E 13 were probed with the antisense murine *QM* riboprobe in order to analyze the pattern of *QM* expression during midgestation (Fig. 2). At the earlier stages of development, analyzed colorimetric product is visible on the surface of the embryo in the cephalic regions overlying the telencephalic vesicle, the diencephalon, the midbrain, and the hindbrain. *QM* transcript was also detected in the muzzle region, and on the dorsal surface of the trunk extending caudally to the tip of the tail. However, the most conspicuous region of *QM* expression was observed in the developing limbs. This intense level of *QM* expression is flanked by a region that appears to be devoid of *QM* message at the site of limb attachment to the trunk. *QM* is expressed in a very broad pattern throughout the embryo at the earlier stages of gestation (E 10), and is downregulated on the surface of the embryo as development proceeds (E 11–13). Expression within the developing limbs remains very high throughout all developmental stages analyzed. The sense strand of the murine *QM* riboprobe was used as a negative control and the resulting embryo was essentially negative for the development of the colorimetric product (data not shown). In addition, a riboprobe specific for murine LEF-1 was used as both a negative and a positive control (data not shown), and the pattern of LEF-1 expression observed was consistent with previously published results [23].

Analysis of the pattern of *QM* protein during murine development by immunohistochemical analysis of tissue sections

In order to detect *QM* protein within internal organs, embryos that had previously been analyzed by whole mount immunohistochemistry were sagittally sectioned and analyzed microscopically. Fig. 3 shows the results of this analysis. This study revealed that while *QM* protein was detected within internal tissues, such as within the lining of the ventricles of the brain (Fig. 3C, D), the most distinctive pattern of *QM* expression was observed within the developing epidermis of the limbs (Fig. 3A, B). Furthermore, *QM* is cytoplasmic (Fig. 3D), consistent with conclusions from previous studies [5, 12].

In some instances, detection of gene expression using whole mount procedures is limited by the lack of reagent penetration into the interior organs of larger specimens. Thus, we also examined tissues that were sectioned prior to immunohistochemical analysis. Embryos harvested from wild-type mice of stages from E 8.5 through E 16.5 were analyzed for *QM* protein by indirect colorimetric immunohistochemistry of tissue sections. Most of the tissues analyzed showed strong immunoreactivity with
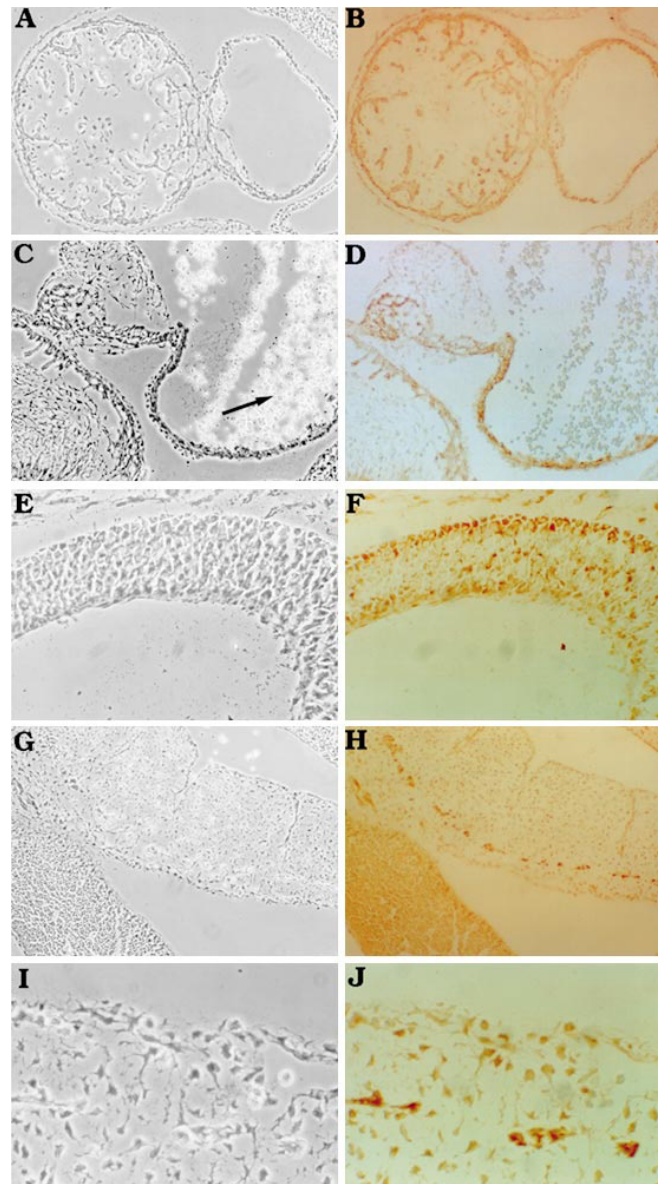

Fig. 4A–J Section immunohistochemistry: *QM* expression during embryogenesis. Embryonic tissue was embedded in polyester wax, sagittally sectioned, and immunohistochemically stained using a *QM* polyclonal antibody. *QM* protein is detected in a broad pattern in many tissues throughout the developing embryo. The heart of the E 9.5 **(A, B)** and the E 11.5 **(C, D)** embryo, the developing otocyst **(E, F)**, and dorsal somites **(G–J)** of the E 9.5 embryo are shown. **A, C, E, G, I** are phase contrast images, while **B, D, F, H, J** are bright field images of the same views shown to the *left* (*arrows* **C–D** red blood cells are devoid of *QM* protein, *arrows* **H—J** cells which express high levels of *QM, S* somites, *NT* neural tube, **A–H** ×100, **I, J** ×400)

the anti-*QM* antibody. However, there were a number of interesting exceptions.

The developing heart expresses *QM* strongly in the E 9.5 embryo (Fig. 4A, B). However, as development proceeds, differentiation of the cardiac myocyte correlates with a downregulation of *QM* (Fig. 4C, D). Nucleated red blood cell precursors within the heart do not express detectable levels of *QM* (Fig. 4B, D). Red blood cells are

Fig. 5A–D Section immuno-
histochemistry: *QM* expression
during embryogenesis. Devel-
oping cartilage of the upper
forelimb **(A)**, humerus **(B)**, ul-
na **(C)**, and digits **(D)** of the E
14.5 embryo are shown. *QM*
expression is strong within spe-
cific zones of developing carti-
lage (**A–C**; *PZ* proliferative
zone, *TZ* transition zone, *HZ*
hypertrophic zone. **A, B, D**
×100, **C** ×400)
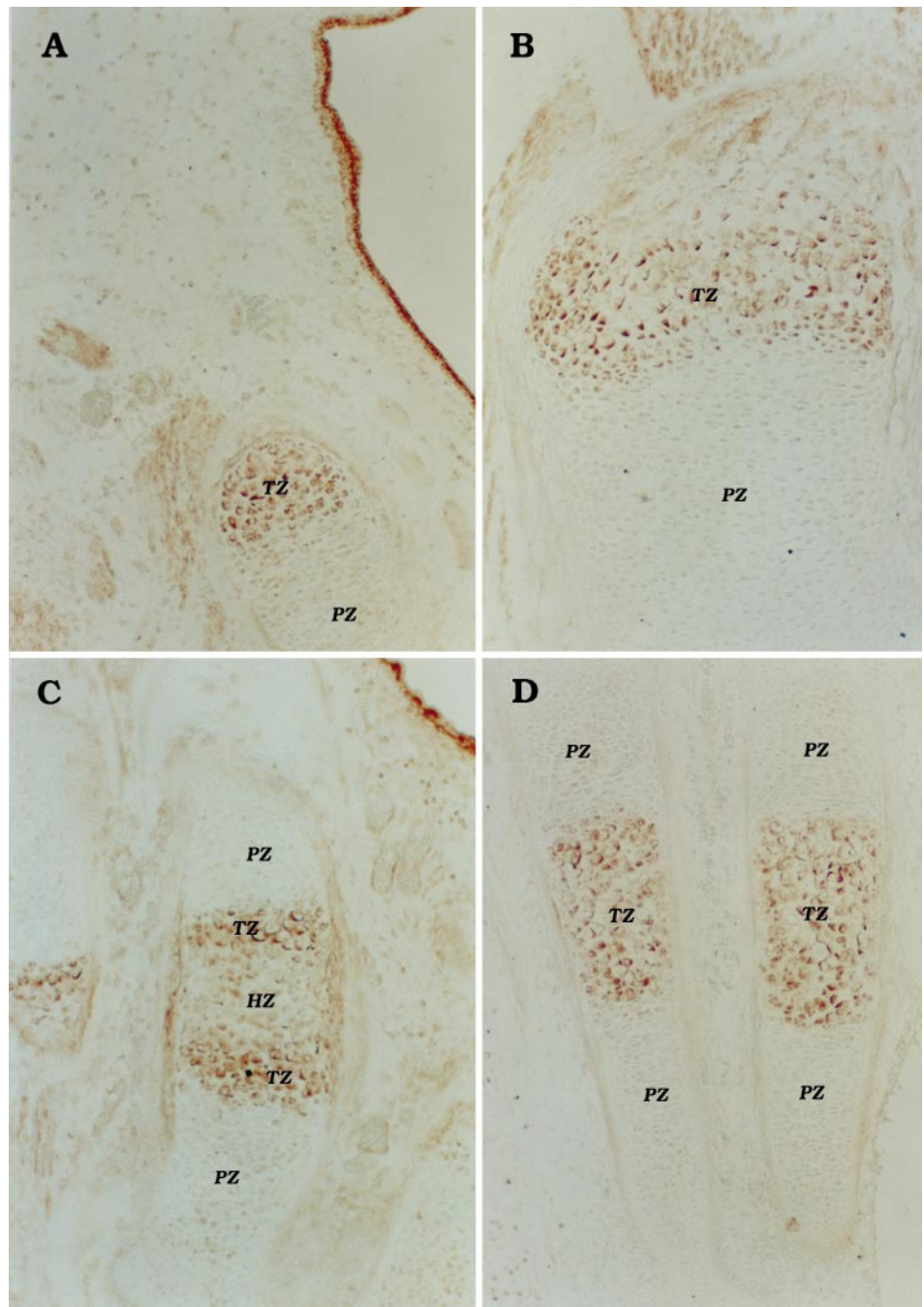
seen in the phase contrast image of the dorsal aorta (Fig. 4A, C), but these cells do not express *QM*, as is evident when the same field is observed under bright field microscopy (Fig. 4B, D). *QM* protein is not detectable in either nucleated red blood cells found in embryos from E 9.5 to 13.5, or in enucleated red blood cells found in older embryos (data not shown).

Immunohistochemical analysis of neural tissue demonstrates that while *QM* is expressed throughout the nervous system (Fig. 4F, H, J), differential levels of expression are observed within some regions, for example in the developing otocyst of the E 9.5 embryo (Fig. 4F). As seen in Fig. 4H and J, a conspicuous line of cells that express high levels of *QM* is observed in the somites of E 9.5 embryos. It is possible that these are migrating cells of neural crest origin moving through the somites. In more medial sections, similar cells have aggregated to form ganglia, and these cells express *QM* strongly.

Chondrocytes within developing cartilage express *QM* at variable levels depending on their state of differentiation. Figure 5 A shows the developing upper forelimb of an E 14.5 embryo in which chondrocytes within the distal region of a cartilage do not express significant levels of *QM*, while more medially located chondrocytes express *QM* at high levels. Similar patterns of *QM* protein

Fig. 6A–D Section immunohistochemistry: *QM* expression in the epidermis of the E 16.5 embryo. Skin of the dorsal trunk **(A)**, whisker pad **(B)**, and limbs **(C, D)** are shown. *QM* is strongly expressed within suprabasal regions of the epidermis and the inner root sheath of developing whisker follicles (**b** basal region, SB suprabasal region, *-ors* outer root sheath, *-irs* inner root sheath, **A–C** ×100, **D** ×400)
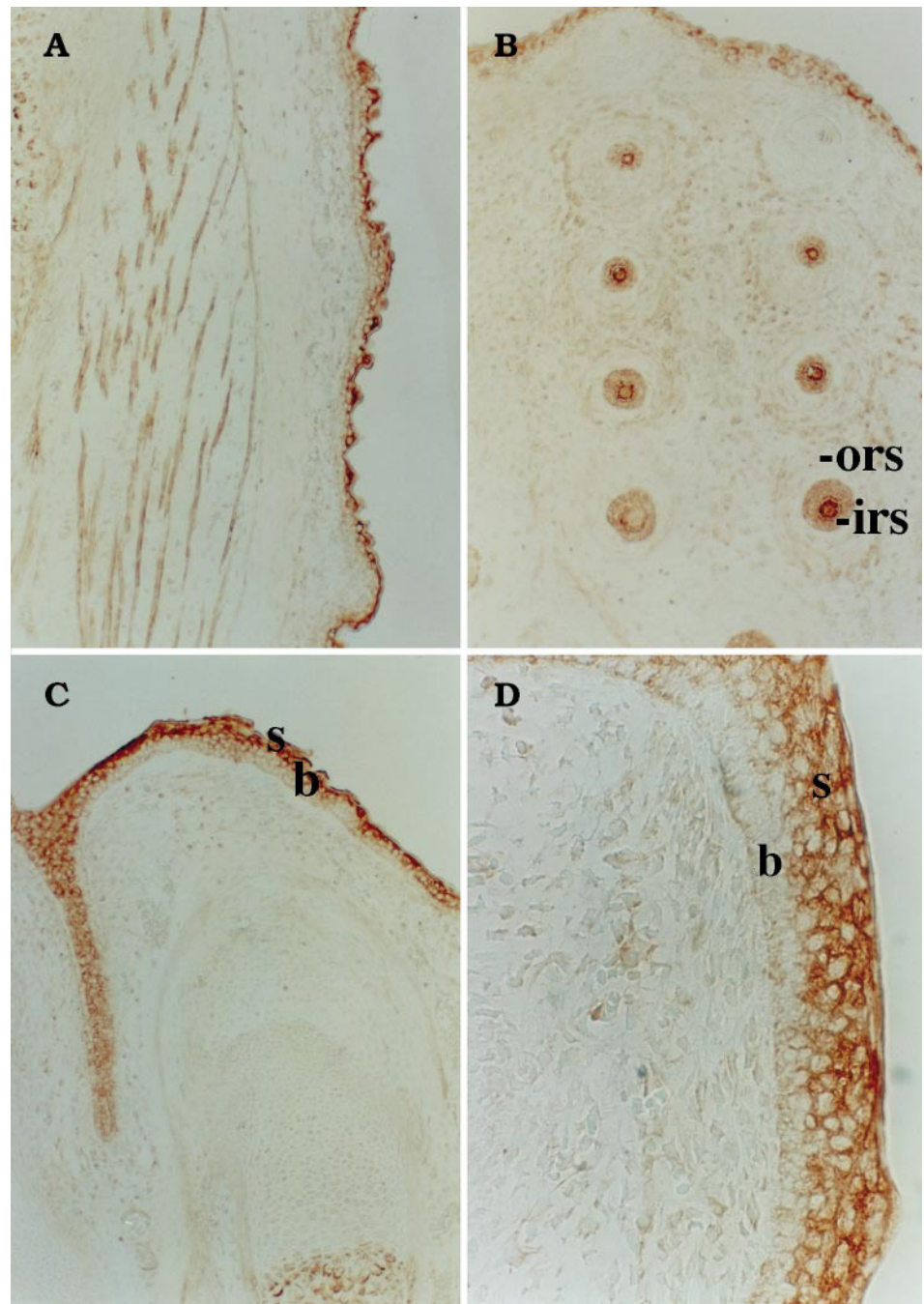
expression were observed in developing cartilage of the humerus (Fig. 5B), ulna (Fig. 5C), and digits (Fig. 5D) of the E 14.5 embryo. Within each of these cartilages, particular zones of chondrocytes express high levels of *QM*, while adjacent regions of the cartilage are apparently devoid of *QM* protein (Fig. 5A–D).

The skin expresses *QM* at all developmental stages examined. In the later stages of gestation, the level of *QM* expression is directly proportional to differentiation; *QM* is expressed within the more differentiated cell. Within the skin of the dorsal trunk (Fig. 6A), the whisker pad region (Fig. 6B), and the developing limbs (Fig. 6C, D), *QM* expression is the highest within the differentiat-

ed, suprabasal keratinocytes. Both the interfollicular keratinocytes of the trunk and limb skin and the follicular skin of the developing whiskers express *QM* within the more highly differentiated keratinocyte.

Analysis of cellular proliferation and the pattern of *QM* expression during murine development

Forelimbs obtained from embryos from E 10.5 through E 16.5 at 1 day intervals were analyzed for cellular proliferation and *QM* expression. Chondrocytes within the developing cartilage of the ulna displayed a striking inverse
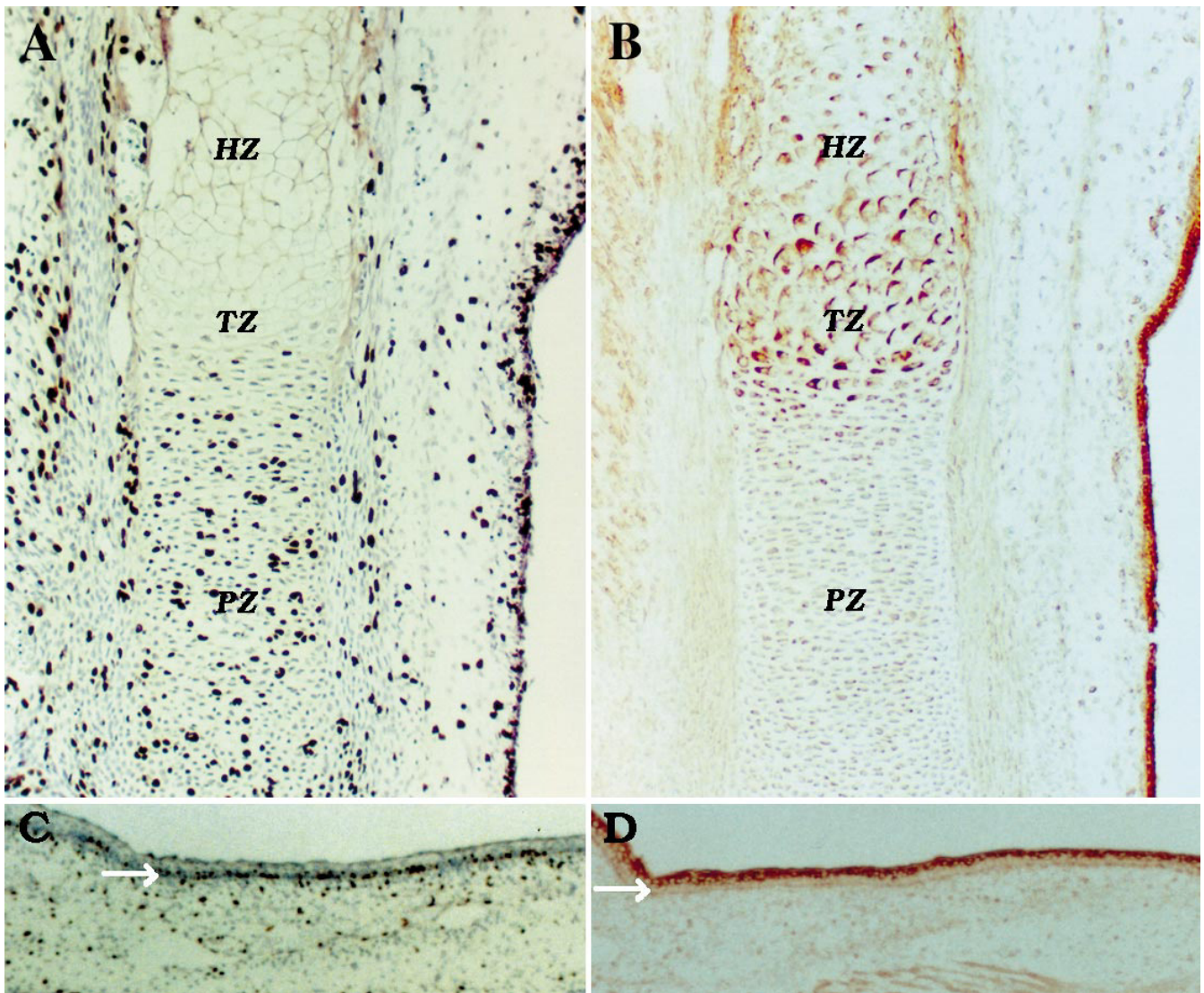
Fig. 7A–D Cellular proliferation versus *QM* expression during differentiation of cartilage and skin. Tangential sections of limbs obtained from E 14.5 embryos were analyzed for in vivo proliferation **(A, C)** or *QM* expression **(B, D)**. Chondrocytes within the proliferative zone (*PZ*) and the hypertrophic zone (*HZ*) of ulna cartilage do not express high levels of *QM* **(A, B)**. In contrast, chondrocytes within the tranzition zone (*TZ*) express high levels of *QM* **(A, B)**. Likewise, proliferative keratinocytes **(C)** within the basal region of the epidermi*s (white arrow)* do not express significant levels of *QM* **(D)**, while suprabasal keratinocytes express high levels of *QM* ×100

correlation between proliferative capacity (Fig. 7 A) and *QM* expression (Fig. 7B). The ulna cartilage expresses *QM* within only a subset of the chondrocytes. Proliferative capacity, however, is absent from the region of the cartilage which is *QM* positive. That these cells were in fact chondrocytes was determined by staining a tangential section with alcian blue, a stain which specifically stains the glycosaminoglycans present within cartilage (data not shown). *QM* is expressed highly within the differentiated, nonproliferative chondrocyte within the transition zone.

Within the epidermis of the skin, an inverse correlation between *QM* expression and proliferative capacity was also evident (Fig. 7C, D). *QM* protein expression was absent or very low within the proliferating basal keratinocytes of the skin (Fig. 7C), while *QM* was expressed at high levels within nonproliferating keratinocytes within the suprabasal regions of the epidermis (Fig. 7D).

Discussion

The pattern of *QM* transcript within the developing mouse

In order to elucidate the function of *QM* in vivo, we have undertaken a detailed study of the pattern of *QM* expression during mammalian development. We have used whole mount techniques combined with conventional immunohistochemistry in order to analyze the global pattern of *QM* expression within the developing mouse.

The technique of whole mount in situ hybridization using a *QM* riboprobe has generated a thorough analysis

of the overall pattern of *QM* gene expression at the level of the transcript during midgestation mouse development. Our analysis revealed that *QM* is expressed within numerous embryonic tissues during midgestation. In the E 10 embryo, for example, *QM* transcript was detected in the surface ectoderm, in a very broad pattern. As development proceeds, however, this pattern of surface expression becomes more restricted. In the E 11 embryo, *QM* is detected within the dorsal midline of the embryo, while a lower level of expression is evident on the ventral surface. This restriction of the pattern of *QM* transcript is even more pronounced at later stages of development. *QM* is expressed very strongly within the developing limbs of embryos of all developmental stages analyzed.

The observation that *QM* is widely expressed within the developing embryo is not surprising since *QM* is expressed in many of the diverse tissues that have been analyzed previously. For instance, expression analysis using RT PCR detected *QM* message in adult murine thymus, liver, intestine, ovaries, uterus, tongue, brain, heart, spleen, lung, skin, kidney, fibroblasts, and embryonic stem cells (data not shown). This wide range of *QM* expression observed during embryogenesis is consistent with results of previous studies using Northern blot analysis [2, 8]. In addition, the apparent downregulation of *QM* that was observed on the surface of the developing embryo is consistent with the reduced level of expression observed in previous studies using Northern blot analysis [2].

The most surprising result of this study, in light of the hypothesized ubiquitous nature of *QM* expression based on limited Northern blot analyses, was that obvious differences in the level of *QM* expression were observed during embryogenesis. The most striking example of this is the conspicuous pattern of *QM* expression which was observed within the epidermis of the developing limbs at all developmental stages studied. This region of the developing limbs which strongly expresses *QM* was flanked by a region in which *QM* is expressed at a much lower level at the site of limb attachment to the trunk. Whether this particular region of the developing embryo is undergoing rapid proliferation, and hence requires high levels of *QM*, is discussed further below.

The pattern of *QM* protein within the developing mouse

In order to correlate the pattern of *QM* expression at the transcriptional level with the pattern of *QM* protein, embryos of various stages of gestation were analyzed for the presence of *QM* protein using the technique of whole mount immunohistochemistry. As was the case for *QM* transcript, *QM* protein was detected in a wide range of tissues within the midgestation mouse embryo. In the E 8.5 embryo, for example, *QM* protein is detected in a broad pattern on the majority of the embryo surface. Only 1 day later, however, *QM* expression is more restricted, and is expressed at a higher level on the dorsal surface of the embryo. This pattern of *QM* expression further corroborates the pattern of *QM* expression as measured by the level of *QM* transcript.

One striking difference was noted, however, between the distribution of *QM* transcript and *QM* protein. Localization of *QM* transcript generated a smooth pattern of staining over the surface of the embryo, while detection of *QM* protein generated a colorimetric reaction product that was ″grainy,″ or ″punctate.″ The reason for this difference was revealed by sectioning embryos after the whole mount procedures. The *QM* protein within the embryo was cytoplasmic, while *QM* transcript was detected throughout the cell. Since the whole mount immunohistochemistry technique produced nuclei that were devoid of colorimetric product while the cytoplasm stained very intensely, a grainy quality was perceived when the entire embryo was visualized.

While the observed cytoplasmic localization of the *QM* protein in vivo was initially surprising in light of the hypothesized function of the *QM* protein as a negative regulator of the nuclear transcription factor *c-Jun* [14], this result is consistent with the results of more recent studies [1, 5, 12]. These studies indicated that not only is *QM* protein localized to the cytoplasm in both yeast and mammalian cell lines, but that the pattern of cytoplasmic staining is punctate, suggesting that the *QM* protein is associated with intracellular organelles. Cell fractionation studies in mammalian cells [12] and in yeast [1] indicate that *QM* is localized to the rough endoplasmic reticulum and is associated with ribosomes.

While the hypothesized function of *QM* as a direct negative regulator of *c-Jun* is inconsistent with the current evidence that *QM* protein is localized to the cytoplasm, the possibility that *QM* localizes to the nucleus under certain cellular conditions cannot be excluded. This scenario has been observed in the case of the transcription factor NF-AT. While normally located within the cytoplasm, upon cellular stimulation and activation of a signal transduction cascade, NF-AT translocates to the nucleus and activates transcription [7]. In our studies, we did not observe any stages of tissues in which *QM* was localized in the nucleus, although we cannot rule out the possibility of nuclear localization under other conditions. However, recent studies in our laboratory have shown that the apparent effect of *QM* on *c-Jun* mediated transcriptional activation of AP-1-containing genes is most likely an experimental artifact [11].

QM expression and cellular proliferation
within developing cartilage and epidermis

The high level of *QM* expression that was initially observed within the developing limbs of the murine embryo using whole mount techniques, prompted a more thorough analysis. In particular, the relationship between cellular proliferation and *QM* expression was addressed within developing limbs. We hypothesized that the high level of *QM* expression observed within developing limbs might be due to an elevated level of cellular proliferation within this region of the embryo. A correlation between the proliferative state and the amount of *QM* ex-

pression has been previously suggested based on several experimental systems. First, serum-starved fibroblasts withdraw from the cell cycle and concommittantly reduce their level of *QM* expression [11]. Upon serum stimulation, the expression of *QM* increases dramatically. Second, various reports have indicated that *QM* expression decreases as differentiation proceeds, and in general this correlates with a lower proliferative capacity [2]. The most convincing of these studies was an 80% reduction in *QM* expression observed during adipocyte differentiation [4]. These studies have suggested that there is a correlation between the degree of cellular proliferation and the level of *QM* expression, and that this relationship may be functionally significant in vivo. Paradoxically, the original isolation of *QM* was based on the higher levels of expression in slower growing, nontumorigenic Wilms' microcell hybrids [2], and in our studies we have demonstrated the opposite correlation between *QM* expression and proliferation.

To analyze the relationship between the proliferative activity of a developing tissue and the level of *QM* expression, embryos were labeled with bromodeoxyuridine, sectioned, and tangential sections were immunohistochemically analyzed for in vivo cellular proliferation and *QM* expression. This analysis revealed a striking inverse correlation between *QM* expression and cellular proliferation within developing cartilage. Within a given cartilage element, several distinct zones were observed. Closest to the ends of the elements are zones consisting of actively proliferating chondrocytes, while adjacent, more medial zones are composed of nonproliferating chondrocytes. Zones of chondrocytes expressing *QM* were adjacent to, but nonoverlapping with, the proliferative zones. Yet, more medial within an element, cells are neither proliferating nor expressing *QM*. In these central zones, the chondrocytes are hypertrophic. Hence, *QM* expression is in the transition zone where cells have ceased proliferation, and before they have undergone hypertrophy. The surprising observation was that the region of proliferation and that of *QM* expression were mutually exclusive; that is, proliferating chondrocytes do not express significant levels of *QM*, and chondrocytes expressing *QM* strongly are not proliferating. Therefore, within developing long bones of the limbs, there is an inverse relationship between the level of *QM* expression and cellular proliferation. This result is the opposite of the result that would have been predicted based on the interpretation of results of previous studies. However, in situ analysis was not part of these studies. In these previous studies, *QM* expression is likely decreased because proliferation is reduced, and, therefore, there are fewer cells making the transition to differentiation. Since *QM* is expressed as cells make the transition to differentiation, this would lead to reduction in *QM* expression concommitant with reduced proliferation.

Endochondral bone formation occurs by a process of ossification after a cartilage template has been established. Differentiation of individual developing cartilages occurs from centers to ends, although among cartilage elements, differentiation is from proximal to distal in a developing limb [3, 21]. Therefore, more proximal cartilages will be more differentiated. Bone forms when proliferating chondrocytes at the end of long bones cease to proliferate and differentiate. These differentiated, hypertrophic chondrocytes form a matrix that is invaded by osteoblasts and nourished by blood vessels during the process of osteogenesis [21]. The transition from the proliferating chondrocyte to the differentiated hypertrophic chondrocyte is a key event in the modulation of bone formation. For example, slowing this transition by overexpression of Indian hedgehog (Ihh) decreases the rate at which chondrocytes differentiate [20]. Ihh is normally expressed in the cartilage of long bones specifically within the transition zone between proliferative and hypertrophic chondrocytes in the chick [20], and mouse limb buds [10, 16]. Thus, Ihh is a key regulator of the transition from the proliferating chondrocyte to the differentiated hypertrophic chondrocyte. Interestingly, *QM* is expressed within the same region and is absent from hypertrophic cartilage and from the proliferative zone. Thus, the pattern of *QM* expression mimics that of Ihh expression. It is interesting to speculate, therefore, that *QM* may play a role in differentiation of chondrocytes during the process of osteogenesis.

QM is expressed in the transition zone between proliferation and differentiation in individual cartilage elements. Cells within this compartment have been reported to cease proliferative activity [13, 17], consistent with results observed in the current study. The differentiated chondrocytes are larger because of cellular hypertrophy, a process which is essential to, and primarily responsible for, the drastic increase in mass of a developing cartilage. The nonproliferating, prehypertrophic cells that express *QM* strongly, have previously been shown to secrete a large amount of protein, primarily glycosaminoglycans. The precise role of *QM* during this process is currently unknown, but one possibility is that *QM* plays a role in the processing or trafficking of proteins which are destined to be secreted.

The inverse correlation between proliferative capacity and *QM* expression is not unique to developing cartilage; this correlation was also observed within the epidermis of the skin. While *QM* expression is absent or barely detectable within keratinocytes of the basal region, a drastic increase in *QM* expression is observed within the suprabasal regions of the skin. Thus, *QM* expression coincides with the transition from the stem cell to the differentiated suprabasal keratinocyte.

Very little is known about the function(s) of the *QM* gene. The original observation that *QM* protein interacts with *c-Jun* protein and inhibits its transcriptional activation activities [14] has been shown by us to be an experimental artifact [11, 12]. Most recently, the yeast equivalent of *QM*, termed *QSR*1, has been shown to associate with the 60 S ribosomal subunit and be involved in subunit joining [5]. The *QSR*1 gene is essential, and temperature-sensitive mutants are defective in protein translation. We have also shown that the *QM* protein associates with ribo-

somes in mammalian cells [12, 15]. Given the extreme evolutionary conservation of *QM* [6] and its role in protein synthesis in yeast, it would be expected that *QM* protein would be expressed in all cells that are synthesizing proteins. Our observations in the developing mouse embryo clearly indicate that this is not the case. Although, as expected, the vast majority of tissues express *QM* protein throughout development there are clear exceptions noted above. One of the most striking exceptions is the hematopoietic cells. We have also shown that bone marrow cells in the adult mouse lack the *QM* protein although they obviously contain ribosomes (A. Mills et al., submitted for publication). Thus, in the mouse and, by inference, in other mammals, *QM* may have function(s) other than an involvement in protein synthesis. It is also possible that *QM* may be involved in a more specific subset of protein translation. These observations in the developing mouse embryo open up powerful new approaches to understanding the function(s) of this evolutionarily conserved protein.

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