Expression of the chicken vimentin gene in transgenic mice: Efficient assembly of the avian protein into the cytoskeleton

YASEMI CAPETANAKI, STEVEN STARNES, AND STEPHANIE SMITH
Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030

ABSTRACT To study expression and function of the vimentin gene, transgenic mice were generated by microinjecting the entire chicken gene plus 2.4 kilobases of 5′ and 2.6 kilobases of 3′ flanking sequences. All the transgenic mice obtained had incorporated multiple copies of the gene. RNA analyses demonstrated that the chicken vimentin gene was efficiently expressed in an appropriate tissue-specific pattern and that the transcripts were properly processed, as in chicken, giving rise to two RNAs. The vimentin transgene was predominantly expressed in lens at levels of up to 10-fold the endogenous level in every transgenic line studied. The chicken vimentin transcripts were efficiently translated into polypeptides that were modified posttranslationally and could assemble into the mouse cytoskeleton. Overexpression of the chicken vimentin gene did not obviously affect the expression of the endogenous gene at the RNA or the protein level. Immunofluorescence microscopy further demonstrated that the chicken protein was properly expressed spatially in lens. However, the levels were much higher in the transgenic animals.

Vimentin shows the most complex pattern of expression of all known intermediate filament (IF) proteins. It is mainly expressed in cells of mesenchymal origin but also found in other undifferentiated cells and cultured cells (1, 2). It is known to be regulated by growth factors in various cell types (3). In most systems, vimentin is down-regulated during development and is partially or completely replaced by cell-type-specific IF subunits (4). It can also be up-regulated, e.g., during avian erythropoiesis (4, 5). Finally, lens (6), avian erythrocytes (7), and certain leukocytes (8) express vimentin as the sole IF subunit in the mature cell. This divergent pattern of the vimentin expression suggests that the regulation of its expression must be complex and its biological function during growth, differentiation, and morphogenesis must be broad and significant. The function(s) of vimentin and other IF proteins remains elusive. A potential role of this protein is to interconnect the nucleus with the plasma membrane (9–12), possibly contributing to communication and transport between the cell surface and the nucleus. However, to date there is no evidence to support functional roles for any of the above structural interactions.

To investigate the expression and function of vimentin, we generated transgenic mice carrying intact or manipulated vimentin genes. Transgenic animals allow us to study vimentin in more than one tissue. We particularly want to study the regulation of the vimentin gene during erythroid, lens, and muscle development and to determine the consequences of altered vimentin gene expression during growth, differentiation, and morphogenesis in these systems where terminal differentiation is linked to vimentin decline and disappearance (13–16). For these purposes, we introduced into the germ line of laboratory mice the fully characterized entire chicken vimentin gene (5, 17). The present work addresses whether the chicken vimentin gene can be expressed in mouse, whether tissue specificity can be observed, and whether the transgenic protein isoforms can be synthesized and incorporated into the cytoskeleton. Analysis of these transgenic mice demonstrated that the chicken gene could be efficiently transcribed in a correct tissue-specific pattern and that transcripts were processed and translated into a protein capable of assembling into cytoskeleton. Overexpression of the transgene did not appear to affect the expression of the endogenous mouse vimentin gene.

MATERIALS AND METHODS

Generation and Screening of Transgenic Mice, VXBK, the chicken vimentin construct used for microinjection, was derived from the genomic λ Charon 4A recombinant λV8 (5) by subcloning in two steps into the pUC18 vector. Thus, the entire gene plus the flanking sequences could be removed from the construct as a unit, free of vector sequences, by digestion with Xho I and Kpn I. Transgenic mice were generated essentially as described (18) with some modifications (19). Tail DNA from 3- to 4-week-old offspring mice, prepared mainly as described (18), were blotted onto nitrocellulose and hybridized to a primer-extended 2.9-kilobase (kb) 5′ HindIII fragment (H3d; Fig. 1) as described (5, 20).

Northern Blot Analysis. Total RNA from various tissues was isolated as described (5, 21). RNA blot analysis was performed as described (5, 20) using a 32P-labeled random-primed (22) chicken cDNA probe (5) or a mouse cDNA probe isolated and sequenced (Y.C., S. Starnes, and K. Rothblum, unpublished results). Final filter washes were done with 0.5× SSC at 50°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

Additional reading:
RESULTS

Generation of Transgenic Mice Containing the Chicken Vimentin Gene. VXBK, the chicken vimentin gene construct used for microinjection (Fig. 1a), is free of vector sequences and contains the entire 7.7-kb vimentin gene (5, 17) plus 2.4 kb of 5’ and 2.6 kb of 3’ flanking sequences. Potentially transgenic mice were screened by Southern blot analysis of tail DNA using a 3’2.9-kb genomic chicken vimentin HindIII fragment (5) (Fig. 1a). In Fig. 1b, all four transgenic founders initially obtained had incorporated multiple copies of the gene, ranging from 3 or 4 (Tg149) to 70–90 (Tg21) copies per haploid mouse genome, in a head-to-tail fashion as is mainly observed in transgenic mice (26, 27). Insert VXBK has a small EcoRI fragment (600–700 base pairs) close to the middle (Fig. 1a). The EcoRI band obtained in the Southern blot (Fig. 1b) is ~12.0 kb and extends from the second EcoRI site of one VXBK molecule to the first EcoRI site of the next molecule in the head-to-tail concatamer. The endogenous vimentin gene has an EcoRI band of ~6.4 kb, easily distinguished from the transgenic band (Fig. 1b, lanes C and D).

Expression of the Chicken Vimentin Gene in Transgenic Mice. To study the expression of the chicken vimentin transgene, founder mice were mated with ICR mice, and total RNA from positive F1 or F2 animals was isolated from various tissues. The expression of the chicken vimentin gene was analyzed by RNA blot hybridization using a chicken cDNA probe (5) that, under the present stringent conditions, could hybridize only with chicken vimentin RNA. Four conclusions emerged from this analysis. (i) The chicken gene was recognized and expressed in the mouse environment. Different lines showed different levels of expression that only partially correlate with the copy number of the transgene. (ii) Comparison between the chicken (Fig. 2B) and the endogenous mouse vimentin expression (Fig. 2C or E) showed clearly that the chicken vimentin transgene was expressed in the correct tissues, brain, heart, intestine, kidney, lens, lung, skeletal muscle, spinal cord, and spleen, but not in blood, liver, or pancreas. This indicates that the integrated construct contains sufficient information to elicit tissue-specific expression. However, the levels of expression relative to endogenous expression varied. For instance, in the brain of mouse Tg21 chicken vimentin was expressed at a higher than expected level relative to other tissues, except lens, reaching at least 3 times the endogenous level. This was not true for other transgenic lines and, consequently, might be an integration-site effect (data not shown). In the other tissues (except lens) of Tg21 mice, however, the expression of the vimentin transgene was lower than the endogenous. Densitometric scanning of x-ray films of RNA blots (Fig. 2), with corrections for different sizes, specific activities of the probes, and exposure times, revealed that, except for lens, spinal cord showed the highest level relative to the endogenous expression (~45%). Next was intestine with ~27% followed by heart (22%), skeletal muscle (20%), spleen (~10%), lung (6%), and kidney (2%). Tg19 mice showed similar patterns of tissue-specific expression but at lower levels (data not shown). (iii) There was overexpression of the chicken vimentin in the lens of most transgenic lines studied. Expression in Tg21 mice reached at least 10 times the endogenous level, expression in Tg19 mice was ~5 times the endogenous level, whereas expression in Tg60 mice was only slightly lower than expression in Tg19 mice. Only Tg149 mice showed lower expression than the endogenous level (20%). It should be noted that Tg21 mice were losing copies during breeding and consequently lost a considerable amount of the initial expression (40–50%). (iv) The chicken vimentin transgene is correctly expressed as two mRNA species (Fig. 2G). The single-copy chicken vimentin gene can produce (5, 28) multiple mRNAs through differential utilization of several polyadenylation sites. Mice produce one 2.1-kb vimentin mRNA (ref. 13 and Fig. 2 C and E), possibly due to the presence of only a single polyadenylation site (Y.C., S. Starnes, and K. Rothblum, unpublished results), as do hamsters (29).
FIG. 2. Expression of the chicken vimentin gene in transgenic mice. Total RNA (10 µg) isolated from various tissues of 4-week-old normal or transgenic mice and 15-day chicken embryos was separated in a 1.3% agarose/formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with a 0.5-kb chicken vimentin cDNA probe (A, B, F, and G) or with a 1.7-kb mouse cDNA probe (C and E). (A) RNA blot from various tissues of a transgenic mouse from family Tg21(F1), hybridized with the chicken probe under high-stringency conditions that do not allow crossreaction with the endogenous (mouse) vimentin at 2.1 kb but do allow reaction with the 2.3-kb and 2.0-kb chicken vimentin bands (exposure time, 15 hr). (B) Same blot as in A exposed for 7 days. (C) Same blot as in B rehybridized to the mouse probe (exposure time, 14 hr). (D) Visualization of the 18S rRNA on the blot (in A–C) by methylene blue staining (RNA loading control). (E) RNA blot from various tissues of normal mice hybridized to the mouse probe (exposure time, 27 hr). Here, only 5 µg of spinal cord RNA was used. (F) RNA blot similar to the blot in E hybridized with the chicken probe under the high-stringency conditions used in A (exposure time, 3.5 days). (G) Lens RNA from all transgenic mice obtained. Lanes: a, Tg21(F1) exposed 35 hr; b, Tg21(F1)* as in lane a but exposed 8 hr; c, Tg21(F3), a third generation subline of Tg21 derived by loss of some of the integrated copies (note the loss of expression from lane b); d, Tg19(F3); e, Tg60(F3); f, Tg149(F1); g, 10-day chicken embryo (chick) (5 µg). Exposure times: lanes c–f, 27 hr; lane g, 18 hr.

Synthesis of Chicken Vimentin and Assembly into the Mouse Cytoskeleton. We then examined whether mammalian cells could translate chicken vimentin mRNA. Lenses from 4- to 5-week-old Tg21 mice were isolated and total protein or an equivalent amount of Triton X-100-insoluble protein was analyzed by two-dimensional gel electrophoresis. Analysis of the gels showed mouse cells synthesized chicken vimentin very efficiently (Fig. 3 D and E). Chicken vimentin can be easily distinguished from endogenous mouse vimentin by its different molecular weight and isoelectric point (see controls in Fig. 3 A–C).

To identify the transgenic protein, we analyzed Western blots of two-dimensional protein gels incubated with chicken vimentin antibodies (24) (Fig. 3 F–J). Comparison of normal chicken vimentin (Fig. 3 F) with the transgenic protein showed no difference. Known multiple isoelectric variants of chicken vimentin (refs. 23 and 30 and Fig. 3 G), generated through posttranslational phosphorylation (31), were similarly expressed in the mouse environment (Fig. 3 I). Thus chicken vimentin synthesized in the transgenic mouse was properly posttranslationally modified, probably by phosphorylation. Furthermore, comparison of the endogenous protein in normal and transgenic lens indicated that overexpression of chicken vimentin in mouse lens does not affect synthesis and stability of the endogenous protein.

Transgenic chicken vimentin subunits are efficiently incorporated into the mouse cytoskeleton, as judged by the Triton X-100-insoluble protein profile (Fig. 3 D and E). To ascertain, however, whether the chicken vimentin formed filaments in mouse cells, we studied its expression, assembly, and structure in leukocytes by indirect immunofluorescence microscopy using specific chicken vimentin antibodies (24). We used single cells to better visualize the assembled filaments. After a long incubation at high titer, the chicken antibody crossreacted slightly with mouse vimentin on a Western blot (Fig. 3, A, H, and I), but this antibody did not crossreact in situ with mouse vimentin, as shown by indirect immunofluorescence microscopy (Figs. 4 D and 5 E). Chicken vimentin was expressed in most leukocytes (macrophages and lymphocytes) as described (8), and it formed filaments similar to normal endogenous vimentin IFs (Fig. 4). However, we do not know if these filaments were assembled from only chicken subunits or contain both mouse and chicken subunits.

Proper Spatial Expression of Chicken Vimentin in the Lens of Transgenic Animals. Vimentin is expressed mainly in the epithelial monolayer of the lens and to a lesser extent in the outer cortical lens fibers but not in the nuclear fibers (14, 15, 32). Using Tg19 and Tg21 mice, we examined the spatial expression of transgenic chicken vimentin by using indirect immunofluorescence microscopy on cryotome frozen sections of lens. Chicken vimentin was correctly localized in the mouse lens and strongly expressed in the whole epithelium (Fig. 5 C′ and D′), similar to expression in normal lens (Fig. 5 G′). Furthermore, in the zone of cell elongation, the bow cells showed strong staining, in both Tg19 and Tg21 mice (Fig. 5 B′ and F′, respectively), similar to the distribution of normal mouse vimentin (refs. 14 and 15 and Fig. 5 A′). The slightly different pattern in the transgenic lens is only due to the stronger staining of the overexpressed vimentin and does not reflect any obviously unusual pattern. Unfortunately, quantitative comparison between normal and transgenic mice cannot be done due to the different fluorescent dyes, but the specificity of the chicken antibodies was confirmed (Fig. 5 E).

**DISCUSSION**

The regulatory sequences required for the tissue-specific expression of vimentin were included in the 12.7-kb VXBK fragment containing 2.4-kb upstream and 2.6-kb downstream flanking sequences used for generating the transgenic mice. The level of expression, however, was variable, between 2%
of the endogenous level in kidney and 1000% in lens. This suggests that the site of integration is crucial for the level of expression rather than the tissue-specificity. In all cases examined, lens expressed vimentin more abundantly than lung, which normally expresses in vivo at least as much vimentin as lens. This strongly suggests either that there is a missing lung-specific enhancer element in the VXBK fragment or that lens has an excess of trans-acting factors for the chicken gene. Indeed, aqueous humor contains platelet-derived growth factor (33, 34) known to up-regulate expression of the vimentin gene (3). It is also possible that a lens silencer is missing from the transgene or that there is lower vimentin mRNA turnover in lens.

During chicken erythropoiesis, the chicken vimentin gene is up-regulated (5); however, during mammalian erythropoiesis, the mammalian gene is down-regulated (8, 13). The present study shows that similar to the endogenous gene, the chicken vimentin transgene was not expressed in mature mammalian erythrocytes (Fig. 2). However, the chicken gene expression was induced during in vitro differentiation of transfected MEL cells (35).

There are a few examples of efficient expression of mammalian genes in mice (36, 37). To our knowledge, no chicken gene, however, has been reported to be expressed at such high levels, or even at the endogenous level, as the chicken vimentin gene. The chicken δ-crystalline gene (38), for instance, did not reach even 0.01% of the endogenous level of any crystalline.

There are some reports where overexpression of a foreign gene decreases the endogenous expression [e.g., of myosin.

---

**Fig. 3.** Analysis of the synthesis and assembly of the chicken vimentin into mouse cytoskeleton by two-dimensional gel electrophoresis. Total cell protein or high-salt-extracted protein from Triton X-100-insoluble IF cytoskeletons from 4- to 5-week-old mouse or 10-week-old chicken lens was separated by two-dimensional gel electrophoresis and stained with Coomassie blue (A–E) or transferred to nitrocellulose and probed with chicken vimentin antibodies (F–I). (A) Normal mouse IF cytoskeleton (one-half lens). (B) Chicken IF cytoskeleton (one-fourth lens). (C) Mixture of material shown in A and B. (D) Transgenic Tg21(F2) mouse IF cytoskeleton (one-half lens). (E) Tg21(F2) mouse total protein (one-half lens). (F–I) Immunoblot autoradiograms of A, B, C, and D (or E) using chicken vimentin antibodies (24). The more-acidic lower molecular weight spots denoted with arrows are degradation products of vimentin as verified by the Western blot analysis (I). A, actin; ChV, chicken vimentin; Mv, mouse vimentin; Cr, crystallins.

**Fig. 4.** Immunofluorescence microscopy of vimentin in normal and transgenic leukocytes. Phase-contrast (A–D) and corresponding fluorescence (A’–D’) micrographs of leukocytes from 6-week-old normal and transgenic TgL9(F3) mice. Normal leukocytes were incubated with mouse vimentin antibodies (A’) or as a control with chicken vimentin antibodies (D’). Transgenic cells were incubated with chicken vimentin antibodies (B’ and C’). Cells in B were focused on the filaments. In C, cells were focused for the phase image and so peripheral filaments around the nuclei are in focus. (×700.)
light chain (37) and T-cell receptor β chain gene (39]). RNA and protein analyses revealed that this is not true for vimentin in transgenic lenses in the present report, suggesting that the trans-acting factors responsible for the vimentin expression in lens are in excess or that the transgene might be constitutively expressed due to the lack of a lens silencer. The present data also indicate that feedback regulation in the vimentin expression in this tissue does not seem to exist.

We thank Dr. Francesco DeMayo and Suzanne Atiee for help during the initial microinjection experiments and JoAnn Julian for her efforts in the two-dimensional gel electrophoresis experiments. We are grateful to Dr. Robert Goldman for the chicken vimentin antibodies. We thank Drs. Joseph Bryan, Constantin Flytzanis, Anthony Means, and Paul Overbeek for critical comments on the manuscript. We also thank the excellent secretarial assistance provided by Suzanne Mascola. This research was supported partly by a National Institutes of Health grant (AR39617-01) and by the Muscular Dystrophy Association, and the American Heart Association Texas Affiliate to Y.C.