Vimentin gene expression during myogenesis: two functional transcripts from a single copy gene

Zendra E. Zehner* and Bruce M. Paterson

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Building 37, Room 4A-21, Bethesda, MD 20205, USA

Received 2 August 1983; Revised and Accepted 10 October 1983

ABSTRACT

We have previously reported that a single vimentin gene is present in the chicken genome (22). In vivo transcription of this gene yields two distinct classes of mRNA's (approximately 2200 and −2500 nts) generated through the possible differential utilization of polyadenylation sites. In this report we demonstrate that 1) both transcripts are functional in vitro and direct the cell-free synthesis of the vimentin polypeptide, as judged by two dimensional gel analysis; 2) three of the four possible adenylylation signals indicated in the sequence of the gene are utilized in vivo as determined by S1 analysis; 3) furthermore, these adenylylation sites are utilized identically in all tissues of the chicken examined; 4) the adenylylation site closest to the body of the message is apparently not used as judged by the S1 conditions employed in the assay.

Levels of vimentin mRNA decrease in different tissues of the chicken during the embryonic to adult transition. There is no evidence for a tissue specific or developmentally regulated pattern of expression for either of the two vimentin transcripts. The same pattern of vimentin mRNA expression is seen in all tissues examined, only the level of expression is altered.

INTRODUCTION

In eukaryotic cells, the cytoskeleton is comprised of intermediate filaments, microtubules, and actin. Within this structural framework the intermediate filament proteins (IFP's) are thought to organize the cytoplasm (1,2), to provide attachment sites for cellular organelles (3), to anchor the nucleus, and to aid in maintaining cell shape (for reviews see refs. 4–7). Based upon their subunit composition, immunological similarity, and tissue distribution, the IFP's have been divided into several classes. The cytokeratins (40–70 kd) are expressed in epithelial cells, the neurofilaments (68 kd, 145 kd, 200 kd) in neurons, the glial fibrillary acidic protein (53 kd) in cells of glial origin, desmin (53 kd) and synemin (230 kd) in certain skeletal, visceral, and smooth muscle cells, and vimentin (54 kd) in cells of mesenchymal origin and most cells grown in tissue culture (5,6).
For the most part, changes in the expression of a specific type of intermediate filament protein parallel developmental transitions seen during tissue differentiation (7). For example, in epithelial cells, 10-20 distinct cytoskeleton proteins have been identified whose pattern of expression is altered during cellular differentiation (8,9). Moreover, it is possible for one cell type to synthesize more than one type of IFP (10,11). This is particularly well illustrated in muscle where the ratio of vimentin to desmin varies during myogenesis (10,12) as well as in different muscle types (6,11,13,14), suggesting a complex regulation for these class members.

We have previously reported that a single vimentin gene is present in the chicken genome (15). In vivo, transcription of this gene yields two distinct mRNA's (approximately 2200 and 2500 nts) generated through the possible differential utilization of polyadenylation sites. In this paper, we have examined: 1) the tissue distribution of these mRNA's; 2) the changes in vimentin mRNA levels during muscle development in vivo and in vitro; 3) the functionality of both mRNA transcripts and 4) the relative utilization of the different polyadenylation sites during myogenesis. Our results indicate that it is possible to produce at least two functional mRNA transcripts from the single vimentin gene and that these transcripts are found in similar abundance in all tissues examined in this study. The potential role of these distinct size classes of vimentin mRNA's with regard to protein cellular localization or function is discussed.

MATERIALS AND METHODS

Vimentin Gene Purification and Analysis

The vimentin gene was isolated and characterized as previously described (15).

RNA Isolation and Analysis

Chicken total RNA was extracted from 14-day embryonic breast muscle and 1-day post-hatch breast, heart or gizzard muscle by guanidine hydrochloride solubilization (16,17,18). RNA extracted from adult muscle (breast and leg) was a generous gift from R. Billiter. RSV-RNA was isolated from fibroblasts transformed with either the Prague-C or Schmidt-Ruppin strain (gift from M. Sobel) of Rous Sarcoma Virus (RSV). Infected primary cultures were passaged three times prior to harvesting. Nontransformed RNA was extracted from mock-treated fibroblast cultures. Poly A+ mRNA was isolated by selective binding to oligo-dT cellulose in the presence of 0.5 M NaCl. Various RNA samples (10-20 μg) were size-fractionated on agarose gels (1%) containing the dena-
The integrity of all the RNA samples analyzed was based upon the mass ratio of the 28S and 18S RNA in the total RNA preparations, as judged by laser scan densitometry. RNA was transferred to nitrocellulose by the Thomas (25) modification of the Northern technique (26). Nick-translated genomic fragments or cDNA inserts were used as probes (27). Blots were hybridized at 42°C (minus dextran sulfate) and washed as described (25). Vimentin enriched mRNA fractions were isolated from total RNA (20 μg) size separated on a methyl mercury hydroxide gel containing low-melting temperature agarose (18). RNA was translated in a micrococcal-nuclease treated, rabbit reticulocyte cell-free protein synthesizing system (19).

Translation products of total RNA and phage selected mRNA were analyzed by SDS-polyacrylamide gel electrophoresis (20) and isoelectric focusing (21,22). Protein products were visualized by autoradiography with Kodak X-OMAT AR film (23).

**Results**

**Identification of the Vimentin gene**

During myogenesis, the synthesis of an abundant class of mRNA's is induced (16) in response to the developing myotube's demand for specific cellular products. cDNA synthesized to myotube mRNA represents an enriched probe for these differentiation specific mRNAs. It can be used to screen directly a chicken genomic library for genes expressed during muscle dif-
Identification of Phage Isolates by mRNA Selection.

Isoelectric focusing of phage selected mRNA translation products. The position of migration of the proteins, actin (arrow A) and vimentin (arrow V) are indicated. Poly A⁺ mRNA translation products are shown on Figure 5, Panel A⁺.

ferentiation. Using this technique we isolated 25 phage containing muscle-expressed gene sequences. The identity of each phage insert was determined by mRNA selections. Fifteen phage selected mRNA coding for a 54 kd polypeptide. Further identification of the 54 kd polypeptide was determined by two dimensional gel analysis. Compared to the standard profile of poly A⁺ mRNA translation products (Fig. 5, Panel 4), these phage selected mRNA coding for a polypeptide identical to vimentin (54 kd) (Fig. 1).

Restriction enzyme digestion of the 15 vimentin phage with Eco RI revealed duplicates and overlaps, decreasing the final number of unique phage isolates to four. These four phage were shown to contain an identical vimentin gene by restriction map profile and heteroduplex analysis (15). Hybridization experiments localized the coding area of the vimentin gene within two Hind III fragments of 1.6 Kbp and 2.6 Kbp. An 8 Kbp Bam HI-Eco RI fragment containing these Hind III fragments and flanking DNA was chosen as
An Eco RI-BAM HI fragment, thought to contain the entire vimentin gene, was subcloned into pBR322. A detailed restriction map of the subclone was determined by the method of Smith et al. (11). The positions of several cDNA plasmids (E8, B3, A5 and G8) within the vimentin gene are noted. The 3'-end of the coding region was determined by nucleotide sequencing (darkened area) whereas the 5'-end of the gene has not been accurately mapped (stippled area). The position of one area of repetitive DNA (hatched box) flanking the vimentin gene is shown. The position of a DNA-S1 probe representing the 3'-end of the vimentin gene is shown. Restriction enzyme sites are depicted as follows: A, Ava II; B, Bam HI; Bg, Bgl II; E, Eco RI; H, Hind III; K, Kpn II; S, Sal I.

In order to assess the level of vimentin gene expression during tissue development, RNA was extracted from the breast muscle of chickens at varying ages (Fig. 3). Equal amounts of total RNA were size fractionated on agarose gels containing methyl mercuric hydroxide, transferred to nitrocellulose, and probed with either the cloned Hind III fragments or the 1.4 Kb insert of the E8 cDNA clone (see Fig. 2). Although the vimentin gene exists as a single copy sequence in the chick haploid genome, two distinct classes of mRNA's were visible on RNA blots (Northern's). These two transcript classes of approximately 2200 (arrow A) and 2500 (arrow B) base pairs in size were equally abundant. With increasing age, the relative proportion of vimentin mRNA within total RNA decreased to 1/8 of its embryonic level as determined by laser scanning of the autoradiogram shown in Figure 3. However, it is clear that adult tissue (3 mths) still contained vimentin mRNA, albeit at reduced levels.
Figure 3. Northern Analysis of Chick Muscle Total RNA. Chick muscle total RNA (10 μg) was size fractionated on a 1% agarose gel containing 10 mM methyl mercuric hydroxide. The RNA was transferred to nitro-cellulose and probed with nick-translated vimentin cDNA plasmid E8 (insert only) as described by Thomas (18). The arrows mark two mRNA species of 2200 and 2500 nucleotides.

A more extensive Northern analysis of various muscle RNA's is shown in Figure 4. Both poly A+ and total RNA contained the same relative proportion of the two vimentin mRNA species. Likewise, in RNA isolated from the breast muscle of a single inbred chicken, both mRNA species were visible (Fig. 4). Thus polymorphism does not account for the different vimentin transcripts seen in outbred chickens. In 1-day hatched chicks there was a reproducible tissue specific difference in the amount of vimentin mRNA present in muscle or heart compared to gizzard (Fig. 4). In comparing myoblast to myotube RNA, there was a consistent increase in the amount of vimentin mRNA exhibited in myogenic cultures (roughly 3 fold). Surprisingly, in RNA isolated from RSV-transformed fibroblasts there also was a consistent increase (2.5-3 fold) in vimentin mRNA compared to RNA isolated from mock-treated fibroblast cultures. This result has been confirmed in two independent infections of primary muscle cultures with either the Prague C or Schmidt-Ruppin strains of RSV.
Figure 4. Northern Analysis of RNA Isolated from Various Tissues During Development.

RNA isolated from 14-day embryonic muscle; 1-day hatched breast, heart and gizzard muscle; cultured myoblasts (nonfused) or myotubes (85% fused); and mock-treated or RSV-transformed fibroblasts were size fractionated on methyl mercuric hydroxide gels as described in the legend to Figure 3.

Functionality of Vimentin mRNA Transcripts

Even though in all the RNA preparations examined both vimentin mRNA transcripts were equally abundant (based on mass), the functional nature of each transcript had not been determined. In order to address this question, RNA was fractionated according to molecular weight on methyl mercuric hydroxide agarose gels cast with low melting temperature agarose. RNA was extracted from each gel slice and translated in a cell-free protein synthesizing system. The resulting autoradiogram (data not shown) marked the position of a 54 kd polypeptide in fractions 19 thru 21. These fractions corresponded to the position of the two vimentin transcripts as judged by Northern blot analysis of the marker lane on the gel (data not shown). The identity of this polypeptide was confirmed as vimentin by two dimensional gel analysis of the cell-free products as shown in Figure 5. Mixing the translation products from the various fractions revealed a single vimentin polypeptide. The faint spot preceding the vimentin peptide probably represents a minor amount.
Figure 5. Translation Product Analysis of Size Fractionated mRNA.

20 μg of total RNA (isolated from 14-day embryonic chicken breast muscle) was size fractionated on a 1% agarose (low-melting temperature agarose) gel containing methyl mercuric hydroxide (10 mM). The gel track was sliced into 3 mm pieces, each slice melted, and the RNA extracted by repeated phenol/chloroform treatment. Following ethanol precipitation in the presence of carrier tRNA, the RNA was translated in a rabbit reticulocyte translation assay. An aliquot of each sample and mixtures of the appropriate samples were analyzed on 2D gels. For reference, translation products of poly A+ mRNA are also shown. The arrows marked A and V mark the position of migration of β-actin and vimentin, respectively. The numbers correspond to the gel fractions analyzed separately or mixed as described in the text.

of phosphorylated vimentin (10%) as has been noted in the literature (11,32). We conclude from these experiments that both vimentin mRNA species are functional and produce a 54 kd vimentin polypeptide.

SI Mapping

Recently, we published the nucleotide sequence of the 3'-end of the
vimentin gene and of the corresponding cDNA clones (15). From this analysis, it was apparent that two sets of tandem polyadenylylation sites approximately 230 nucleotides apart, containing the canonical sequence AATAAA (34,35), were present within the 3'—nontranslated region of the vimentin gene (for sequence see Fig. 7). Three cDNA clones (Fig. 2) E8, A5 and G8, ended after three of the four possible adenylylation signals. However, only clone G8 had retained a portion of the 3' poly A track during construction of the cDNA library. In order to map the functional end-points of in vivo vimentin transcripts, we conducted SI mapping studies on both embryonic poly A+ and total RNA (Fig. 6A). For these experiments a Bgl II-Pvu II fragment of 550 base pairs was used labeled at the Bgl II site (see Fig. 2). This probe extends 150 nts past the last adenylylation site within the vimentin gene. As can be seen in Figure 6A, three DNA fragments labeled A, B, and C are protected by vimentin mRNA. These fragments correspond to mRNAs terminated within 20 base pairs of polyadenylylation sites 2, 3, and 4, respectively, as shown in Figure 7. Since no transcript terminating just after adenylylation site 1 was seen (Fig. 6B) we assume the site is not used, or alternatively, the hybrid is not stable under the SI assay conditions used. Control experiments in the absence of SI digestion (lane a) or in the presence of mouse RNA (lane b) reveal only reannealed probe. The slight heterogeneity in the length of the protected DNA fragment is due to the inability of the SI nuclease to remove precisely the single-stranded overhang.

Similar SI digestion experiments were conducted on several different types of RNA extracted from muscle at various times during myogenesis (Fig. 7B). The relative amounts of the two transcripts seen on Northern blots (Figs. 3 and 4) were similarly reflected in 3'-end protection experiments: less vimentin mRNA is produced in adult tissues compared to embryonic muscle (lanes j-p compared to lanes e-f). Even in adult tissues, where vimentin synthesis is low, SI analysis revealed a low level of the three mRNA transcripts seen in embryonic tissues and in cultured material. These bands (lane j-p) are distinct on longer exposures. Likewise, in RSV-transformed fibroblasts, the amount of protected vimentin mRNA (lane h) is increased relative to mock-treated cultures (lane i). Additional SI experiments were conducted with a second probe using the complete kinased 1 kb Bgl II fragment without the secondary Pvu II digest. In this case, no longer vimentin transcripts could be seen in embryonic or 1-day hatched muscle total RNA, or in poly A+ (lanes r-u). Therefore, we conclude that there are no additional polyadenylylation sites for at least 500 nucleotides downstream from site 4.
Figure 6. SI Mapping Experiments of Vimentin mRNA Transcripts.
A. Total RNA was heteroduplexed to $^{32}$P-labeled Bgl II-Pvu II fragment encompassing the 3'-end of the vimentin gene (see Fig. 2 for position of the SI probe). Single-stranded DNA was removed by digesting with SI (1000 U) for 1 hr at room temperature. The size of RNA-protected DNA fragments was determined on an 8 M urea-6% polyacrylamide sequencing gel (21). In lane b monkey RNA (7 µg) was used as a control. Lane c and a contained embryonic total RNA (7 µg) plus or minus the addition of SI. Embryonic poly A+ mRNA (3 µg) was used in lane d. A $^{32}$P-labeled Hpa II digest of pBR322 was used as molecular weight markers (lane P). Arrows A, B, and C mark the size of RNA-protected DNA fragments.
B. Various RNA samples (total RNA, 7.5 µg; poly A+ mRNA, 2.5 µg) were analyzed by SI digestion with two overlapping DNA probes. Probe 1 (lanes e-q) was described in Fig. 6A. Probe 2 (lane r-u) was the entire Bgl II fragment prior to secondary cutting with Pvu II to generate probe 1. The lanes marked λ and P are molecular weight markers generated by a Hind III digestion of λ and a Hpa II digestion of pBR322, respectively. SI digestion products were sized on a sequencing gel as described in A. Total RNA was utilized in all SI experiments unless noted otherwise. RNA samples analyzed are as follows: 14-d embryonic breast muscle, lanes e, r and t; 14-day embryonic poly A+ mRNA, lanes f and s; monkey RNA, lanes g and u; RSV-transformed fibroblast, lane h; mock infected fibroblasts, lane i; adult AID muscle, lane j; adult upper breast muscle, lanes k and l; adult leg muscle, lane m; adult lower breast muscle, lane n; 1-day hatched breast muscle, lane o; 1-day hatched gizzard, lane p; and 1-day hatched heart, lane q.

In all the RNA's tested using either probe, the relative utilization of the three adenylylation sites 2, 3, and 4, was approximately equivalent.

By comparing the sequence of the DNA probe and the length of the SI protected fragments on the same sequencing gel, it was possible to determine the approximate site of poly A addition for transcripts A (site 2) and B (site 3). These sites are underlined in Figure 7. Due to the heterogeneity of the SI digestions and a stretch of A residues following site 4, it was impossible to map the end of transcript 'C'. It should be noted that the presumed ends of the cDNA clones did not match with the poly A addition sites as determined by sequencing of the SI protected fragments. In this case the ends of the cloned cDNA may have been generated by SI 'nibbling' during construction of the original cDNA library. The lack of poly A tracks in the sequence of the cDNA clones support this interpretation (15).

DISCUSSION

We have previously shown there is one copy of the vimentin gene per haploid genome in the chicken, yet transcription produced two major size classes of vimentin mRNAs (15). By electrophoretically separating the mRNAs under denaturing conditions, we have succeeded in resolving the two distinct size classes of vimentin mRNA transcripts. Each size class of messenger RNA was functional in an in vitro translation assay and directed the cell synthesis of the vimentin polypeptide, as judged by 2D gel analysis.

The vimentin cDNA plasmid (E8) was used as probe to quantitate the amount of vimentin mRNA produced in different muscle tissues during development (Northern blot analysis). Currently, a conflict exists in the literature as to whether or not adult muscle tissue contains vimentin. Using immunofluorescent staining techniques employing specific, hyperimmune sera (36,37),
Figure 7. Sequence of the 3'-end of the Vimentin Gene.

The complete nucleotide sequence of the 3'-nontranslated region of the vimentin gene is shown. The four possible signal sequences (AATAAA) are numbered in succession. Functional adenylation sites as determined by sizing and nucleotide sequencing of S1 protected DNA:RNA fragments are underlined. The end positions of relevant cDNA plasmids are likewise designated with their corresponding names. BamHI II denotes the site of labeling for DNA-S1 probes used in Figure 6.

several investigators have attempted to compare the cellular content and location of the IFPs, desmin and vimentin in muscle. Using this approach, Lazarides et al. has reported the relocation of vimentin from the myoblast cytoskeleton to the Z-disc in the developing myotube (36). Concomitant with muscle differentiation, desmin is synthesized and, as judged by immunofluorescent staining, desmin is localized only in the Z-disc region. Employing similar methods, Holtzer et al. also reported that desmin appears to localize in the Z-disc, but found no staining of this structure with anti-vimentin (37). A direct assay for the presence of vimentin mRNA in adult muscle tissue by Northern blot analysis and S1 mapping, suggests vimentin is indeed expressed in both developing and adult muscle, albeit at different levels. However, the question of cellular localization remains open.

On Northern blot analysis, we detected a reproducible increase in the amount of vimentin mRNA produced in RSV-transformed fibroblasts compared to
mock-treated cultures (Fig. 4). This result was obtained with two different strains of RSV and was confirmed in Sl mapping studies (Fig. 6). On the other hand, the level of actin mRNA is reportedly unaffected by RSV transformation, as judged by Northern blot (personal communication, A. Schmidt and B. de Crombrugge). In this context, RSV-transformation of chick embryo fibroblasts is thought to decrease the expression of tropomysin (38), fibronectin (39) and collagen (40) genes, yet activates the transcription of embryonic globin genes (41). Interestingly, vimentin is one of the few minor proteins specifically phosphorylated by the pp60src-kinase in vivo (42). We have assayed (by Southern blots) the repetitive DNA flanking the vimentin gene for homology to the RSV-LTR and have found no evidence of sequence homology. At this time a possible mechanism for the RSV induction of vimentin gene transcription is unclear. We wish to stress that the quantitative aspects of vimentin gene transcription are preliminary in nature as there were no internal standards included for either the Northern blots or Sl gels. However, these changes in the level of vimentin mRNA are highly reproducible and, therefore, likely reflect vimentin mRNA levels in the RNA preparations.

The nucleotide sequence at the 3'-end of the vimentin gene revealed multiple adenylylation sequences. A total of four such sites containing the canonical sequence (AATAAA) are arranged in tandem pairs. The distance between these pairs of sites (230 nts) roughly corresponds to the size difference between the two classes of vimentin mRNA molecules exhibited on Northern blots (Figs. 3 and 4). We have assessed the functional end points of vimentin mRNA synthesized in vivo and demonstrate three of the four poly A sites are utilized (Figs. 6 and 7; sites 2, 3, and 4). No transcript was found to end after site 1. Although a previously characterized cDNA plasmid (E8) did end between site 1 and 2 (15), this plasmid contained no poly A tract. It is likely this 3' end was artificially generated during the construction of blunt-ended, double-stranded cDNA which was subsequently cloned into pBR322.

We have searched for a tissue specific utilization of the various adenylylation sites. In RNA extracted from different muscles (breast, leg, heart or gizzard), or at various stages during development (14-day embryonic to 3 month breast muscle), we have found no preference for a specific polyadenyllylation site. Despite the varying levels of synthesis of vimentin mRNA reported above, the relative proportions of transcripts terminating after sites 2, 3, and 4 were relatively constant. No transcript was ever detected from site 1. A comparison of the nucleotide sequence surrounding site 1 shows no remarkable
difference to that of the other sites, therefore, it is not apparent why site 1 is nonfunctional.

We have determined the site of poly A addition for site 2 to be near a GA and/or GT dinucleotide, and for site 3, a GC dinucleotide (Fig. 7). On a Northern blot the amount of vimentin mRNA terminated at site 2 roughly equals that of the larger mRNA species terminated at sites 3 and 4. The SI analysis suggests site 3 is the preferred adenylation signal for the longer transcripts since the intensity of band B is 3-4 times that of A and C. By comparison to other sequences reported in the literature, there seems to be considerable flexibility in the site of poly A addition (43-46). To date no canonical sequence other than the initial AATAAA recognition site has been confirmed to direct mRNA adenylylation.

Recently, other single copy genes, namely calmodulin (43) and B2-microglobulin (44), have been isolated which also produce several mRNA's by the differential utilization of multiple adenylylation sites. In both these cases three mRNA's are produced and the 3'-end arrangement of these genes greatly resembles that of vimentin wherein two sets of adenylylation sites exist approximately 250 nts apart. The function of these multiple mRNA's is as yet unknown. We can only speculate that the various transcripts might be utilized differently within the cell; each transcript may be compartmentalized in accordance with the different information encoded in the 3' portion of the mRNA sequence.

Recently, a cDNA plasmid for hamster vimentin has been isolated and sequenced (47). In agreement with our results, this plasmid hybridizes to several mRNA bands by Northern analysis. When the same probe is hybridized against hamster DNA (Southern blots) few hybridization bands are detected. Although no data is presented on the copy number of the hamster vimentin gene, it is tempting to speculate that mRNA complexity observed may reflect multiple adenylylation sites at the 3'-end of the hamster vimentin gene(s). We have isolated the human vimentin gene and intend to characterize the nature of the transcript(s) produced. This should allow us to determine whether or not multiple mRNA's are the general property of vimentin genes or if our results reflect a particular case.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. W. M. Holmes and Dr. M. C. O'Neill for their support and suggestions, and C. T. Mock for his expert assistance in photography. Z. E. Z. was supported by a post doctoral fellowship from the Muscular Dystrophy Association of America.
REFERENCES