Expression of the Genes Encoding the Cytoskeletal Proteins Vimentin and Protein 4.1

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1987

(Submitted May 18, 1987)

For my parents,

and for Lisa

Acknowledgements

There have been so many people who have made my years at Caltech both rewarding and unforgettable, I hardly know where to begin. But try I must. Elias Lazarides, my advisor, provided an intense and stimulating environment for research and learning. I am grateful to Elias for his trust and confidence in me, allowing me to follow my nose and see what would turn up. Several people in the lab contributed greatly to my education, scientific and otherwise. Bruce Granger showed me excellence by example. Bruce was always a tough act to follow, both in the lab as well as during our many hypoxic scrambles up too-tall mountains. From Randy Moon I learned many things, including the value of scientific debate, argumentation, and other excuses for having a beer (or two). Jim Nelson introduced me to the fine art of cell culture, and to the not-so-fine art of massive sushi consumption. I would like to thank my collaborators over the past several years, Semie Capetanaki, Jeff Stack, and Craig Bond, whose input and influence on my research is reflected in the work I present here. Ilga Lielausis and Jennie Cortenbach kept me going with their tireless and unfailing preparation of various and sundry growth media and supplies, not to mention their finely honed maternal instincts. Over the past year or so, Anne Rafferty made sure I was a happy cell culturer. I thank Valerie Purvis for her artistic illustrations and graphics. All the ladies in the office helped me unscramble seemingly undecipherable writing into coherent, typed English. I am especially grateful to Stephanie Canada, whose sharp eyes and nimble keyboard fingers helped me produce manuscripts, not to mention this thesis, in record time.

I appreciate the interest that my thesis committee has shown in my science. I thank Elliot Meyerowitz for his incisive suggestions and observations, Mel Simon for his insightful enthusiasm, and Norman Davidson for sharing some of his invaluable experience with me. To simply thank Barbara Wold as a member of my committee would be inadequate. Over the past three or four years, Barbara has been a continuing source of counsel, encouragement, and knowledge, for which I am more than just grateful.

During my graduate years at Caltech, I was supported by predoctoral fellowships from the National Science Foundation and Gordon Ross Medical Foundation. I especially thank Dr. Ross for his firm commitment to scientific education. Parts of the cost in the preparation of this thesis were defrayed by the Jean Weigle Memorial Fund.

ABSTRACT

The investigations presented in this thesis represent an effort to understand the regulated expression of cytoskeletal proteins in differentiating cell systems. Vimentin is an intermediate filament protein whose expression is regulated during the differentiation of a variety of cell types. I have isolated DNA probes specific for chicken vimentin and utilized them for the study of vimentin gene regulation. The single chicken vimentin gene encodes multiple mRNAs that differ in the lengths of their 3' untranslated regions. These mRNAs are differentially expressed in a tissue-specific manner. Furthermore, vimentin mRNA increases to high levels during chicken embryonic erythropoiesis, underlying similar changes in vimentin protein accumulation.

Unlike nucleated avian erythrocytes, mammalian erythrocytes are devoid of intermediate filaments. I show that cultured murine erythroleukemia (MEL) cells repress the levels of vimentin mRNA during inducer-mediated differentiation, resulting in a subsequent loss of vimentin filaments. The expression of vimentin in these cells reflects the disappearance of vimentin filaments during mammalian erythropoiesis in vivo. To examine the molecular basis for divergent vimentin gene regulation in avian and mammalian erythropoiesis, I have studied the behavior of chicken and hamster vimentin genes introduced into MEL cells. During MEL cell differentiation, RNA encoded by transfected chicken vimentin genes significantly increases in abundance, whereas RNAs arising from either transfected hamster vimentin genes or the endogenous mouse vimentin gene are repressed. The results suggest that the difference in vimentin expression in avian and mammalian erythropoiesis is due to a divergence of *cis*-linked vimentin sequences.

Protein 4.1 is an extrinsic membrane protein that facilitates the interaction of spectrin and actin in the erythroid membrane skeleton. Previous studies have shown that chicken protein 4.1 exists as a multiplet of related polypeptides that are differentially expressed during erythropoiesis. I have isolated cloned cDNA probes for chicken protein 4.1, and have found that a single protein 4.1 gene encodes multiple mRNAs by differential processing; the ratios of protein 4.1 mRNAs change during erythroid development. In vitro translation experiments demonstrate that while the expression of protein 4.1 polypeptides is specified initially at the mRNA level by RNA processing, the ultimate expression of protein 4.1 variants is further determined translationally.

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CHAPTER 1:

INTRODUCTION

Patterns of cytoskeletal protein expression have provided several model systems in which to study the regulation of gene expression and morphogenesis during cellular differentiation. In multicellular organisms, the maturation of distinct cell lineages yields differentiated cell and tissue types capable of performing specialized functions. The functional specificity of any given cell is necessarily a reflection and manifestation of its structure and organization. For example, the force-generating capability of a striated muscle cell is due to a highly ordered arrangement of multiple units of the fundamental contractile apparatus, the sarcomere. Similarly, the capacity of erythrocytes to carry oxygen to systemic tissues through narrow and tortuous capillary beds is facilitated by their cell membranes' deformability and high tensile strength. An advantage of studying differentiating cell systems is presented in the opportunity to correlate changes in gene expression with changes in cell structure and function. Through genetic and molecular manipulations, such correlates can be tested directly by altering the developmental expression of genes that would be otherwise regulated as part of a differentiation program. In this thesis, I have examined the developmentally-regulated expression of two cytoskeletal protein components. The first, vimentin, is one member of the intermediate filament protein family, which by morphological criteria appears to play a structural role in the cytoplasm of higher eukaryotic cells. The second, protein 4.1, is a component of the submembranous network of proteins initially and extensively characterized in erythroid cells, and has been shown to facilitate and modulate the interaction of other components in this network. I will highlight here the relevant information that has served as the foundation for the investigations presented in this thesis.

Intermediate Filaments

Intermediate filament proteins comprise a heterogeneous family of cytoskeletal proteins that display a characteristic 10 nm-diameter filament morphology. By immunological, biochemical, and protein and DNA sequence criteria, five distinct classes of cytoplasmic intermediate filament proteins have been defined in higher vertebrates. The keratins are expressed in epithelial cells, desmin in smooth and striated muscle, glial fibrillary acidic protein (GFAP) in glial cells, neurofilaments in neuronal cells, and vimentin in a wide variety of cell types, and often in immature stages of cell differentiation (reviewed by Lazarides, 1982; and Steinert et al., 1985). All intermediate filament subunit types share a highly conserved α-helical central rod domain structure of 310-340 amino acid residues, flanked by variable subunit-specific non-helical head- and tail-pieces (Geisler and Weber, 1982; Hanukoglu and Fuchs, 1982, 1983; Quax et al., 1983, 1985; Lewis et al., 1984; Lewis and Cowan, 1985; Steinert et al., 1985; Zehner et al., 1987). The characteristic 10 nm filament array is believed to be due to the polymerization of subunits via a tetrameric organization of the conserved central rod domains in coiled-coiled dimers (Geisler et al., 1982; Geisler and Weber, 1982; Hanukoglu and Fuchs, 1982; Aebi et al., 1983; Steinert et al., 1983), which probably is influenced by the variable non-helical domains (Geisler and Weber, 1982). The presumed cell type-specific functions of intermediate filament subunits, inferred by their tissue-specific expression, most likely reside in the variable non-helical flanking domains, which probably are disposed radially at the peripheries of the filament structure (Fraser and MacRae, 1983; Steven et al., 1983a,b).

A specific intermediate filament subunit often is induced as part of the terminal differentiation program in a variety of cell lineages. For example,

vimentin is expressed as the major intermediate filament protein in fibroblasts (Franke et al., 1978), lens fibers (Bradley et al., 1979; Ramaekers et al., 1980), and avian erythrocytes (Granger et al., 1982). In other cases, vimentin is present during immature stages and precedes the appearance of the cell type-specific subunit. During terminal differentiation, the particular subunit may partially or completely replace vimentin as the major intermediate filament protein. In developing chick spinal cord, neurofilament protein is expressed in lieu of vimentin during the terminal differentiation of neurons (Tapscott et al., 1981a,b). In chicken myogenesis, vimentin is co-expressed with desmin, whose synthesis is induced following fusion of myoblasts in the formation of multinucleate myotubes (Bennett et al., 1979; Gard and Lazarides, 1980); the co-expression of vimentin and desmin persists in adult chicken skeletal muscle (Granger and Lazarides, 1979). Heterogeneity of intermediate filament composition within a cell type is also exhibited in certain mouse retinal neurons, where vimentin co-exists with neurofilament protein (Dräger, 1983), and in astrocytes, where GFAP and vimentin are both present (Tapscott et al., 1981b; Yen and Fields, 1981; Schnitzer et al., 1981). Interestingly, the 70,000 dalton core neurofilament polypeptide (NF70) has been found in sub-stoichiometric quantities relative to vimentin in erythrocytes from chicken embryos and young chicks (Granger and Lazarides, 1983). The coexistence of vimentin with desmin, GFAP, or neurofilament protein results in a heteropolymeric intermediate filaments (Gard et al., 1979; Gard and Lazarides, 1980; Steinert et al., 1981; Quinlan and Franke, 1982, 1983; Sharp et al., 1982; Ip et al., 1983; Granger and Lazarides, 1983).

The cell type-specific expression of intermediate filaments suggests that each subunit type performs a specialized function tailored to the requirements of

the differentiated cell. Studies of intermediate filament protein expression and intracellular localization suggest a structural role for these elements. During chicken skeletal myogenesis in vitro, there is an induction of desmin synthesis and accumulation upon fusion of immature myoblasts into multinucleate myotubes (Gard and Lazarides, 1980). This induction is manifested by the appearance of cytoplasmic desmin-containing filaments (Bennett et al., 1979; Gard and Lazarides, 1980). During later stages of myogenic differentiation, desmin redistributes from a cytoplasmic filamentous pattern to a sarcomeric Z-line association at a time when adjacent sarcomeres become aligned (Bennett et al., 1979; Gard and Lazarides, 1980). In adult chicken skeletal muscle, desmin is localized at the peripheries of Z-discs (Granger and Lazarides, 1978). One interpretation of these observations is that desmin or desmin-containing filaments initially facilitate, and subsequently maintain the cross-striated sarcomeric registry in skeletal muscle by redistributing to the Z-disc peripheries and interlinking adjacent myofibrils across the myocyte cytoplasm (Granger and Lazarides, 1978, 1979; Gard and Lazarides, 1980). Vimentin follows the same redistribution pattern as desmin during myogenesis, but is present in mitotic and post-mitotic myoblasts, as well as in fusing and post-fusion myotubes (Granger and Lazarides, 1979; Gard and Lazarides, 1980).

The mechanism by which intermediate filament redistribution occurs during myogenesis is unknown. Desmin and vimentin may associate preferentially with Z-discs due to the de novo appearance of binding sites at these structures. Another possibility involves the developmentally-regulated expression of Z disc-specific desmin and vimentin mRNAs, whose products would localize only at Z-discs. Posttranslational modifications of desmin and vimentin also may affect their subcellular localizations and interactions. Lastly, the ratio of vimentin to

desmin may influence intermediate filament distribution. To distinguish among these possibilities, studies were carried out to examine the accumulation of desmin mRNA (Capetanaki et al., 1984) and vimentin mRNA (Capetanaki et al., 1983 [Chapter 2 of this thesis]) during myogenesis in vitro, using cloned DNAs as probes. The major increase in desmin mRNA accumulation occurred during the time of the major onset of myoblast fusion, coinciding with the time of increased desmin protein synthesis (Gard and Lazarides, 1980; Capetanaki et al., 1984). The specific expression of desmin during and subsequent to myoblast fusion therefore appears to be regulated primarily at the level of mRNA abundance. The induction of desmin mRNA levels and desmin synthesis parallels the induction of other muscle-specific mRNAs and proteins at the time of fusion (Devlin and Emerson, 1979; Shani et al., 1981; Moss and Schwartz, 1981). Vimentin mRNA and vimentin protein synthesis were maintained at fairly constant levels during myogenesis (Gard and Lazarides, 1980; Capetanaki et al., 1983 [Chapter 2]). Since both desmin and vimentin each exist as a single copy per haploid avian or mammalian genome (Zehner and Paterson, 1983; Capetanaki et al., 1984; Quax et al., 1983, 1985), each producing only one protein-coding sequence, it seems unlikely that a switching of desmin or vimentin mRNAs (and hence, the respective protein products) occurs. The relatively constant levels of vimentin and desmin protein at the time of myogenic intermediate filament redistribution renders unlikely the possibility that changing ratios of subunit composition affect localization. It is possible that the filament redistribution in fact is mediated solely by a specificity conferred by desmin, and that vimentin passively co-localizes in desmin-vimentin heteropolymeric filaments.

Both vimentin and desmin are substrates for cAMP-dependent protein kinases (O'Connor et al., 1981; Gard and Lazarides, 1982), and at the time of

myogenic intermediate filament redistribution, the sensitivities and sites for these kinases change (Gard and Lazarides, 1982). Furthermore, Danto and Fischman (1984) have characterized a monoclonal antibody that recognizes desmin in adult cardiac and skeletal muscle, but not in cultured cardiac muscle cells or certain regions of embryonic myocardium. The differential reactivity to this antibody presumably reflects conformational differences or differential masking, which only allows this epitope to be reactive in adult muscle (Danto and Fischman, 1984). Together these results suggest that the redistribution of desmin- and vimentin-containing filaments during myogenesis is effected either by posttranslational modifications of these proteins, by the elaboration of a vimentin- or desmin-specific Z-disc receptor, or both. However, the levels of desmin and vimentin accumulation appear to be determined primarily at the level of mRNA abundances.

The highly ordered striations of cardiac and skeletal muscle cells, quite obvious to the microscopist's eye, is perhaps an extreme example of cytoplasmic organization. The observations discussed above suggest that desmin- and vimentin-containing intermediate filaments play a role in establishing and maintaining this organization. Intermediate filaments have also been implicated in nuclear anchorage and nuclear centration. In cultured human fibroblasts, intermediate filaments appear to span the cytoplasm and interact directly with the nuclear laminar residue following detergent extraction (Lehto et al., 1978). Centrifugal enucleation of cultured fibroblasts reveals a residual connection of intremediate filaments between the extruding nucleus and the presumptive cytoplast, further suggesting an anchoring role for these filaments (Laurila et al., 1981). However, other studies have shown that a disruption of intermediate filaments by microinjection of monoclonal antibodies has no obvious effects on the

morphology of cultured fibroblasts (Lin and Feramisco, 1981). The relevance of this observation to other cell types and differentiating cell systems remains to be determined. In the nucleated erythrocyte of non-mammalian vertebrates, the centrally-located nucleus appears to be anchored by a filamentous network (Harris and Brown, 1971; Cohen, 1978). The filaments of this system are 10 nm in diameter, and appear to span the cytoplasm and attach to the plasmalemma and nucleus (Harris and Brown, 1971; Virtanen et al., 1979; Woodcock, 1980; Granger and Lazarides, 1982). Immunological and biochemical analyses have demonstrated that the transcytoplasmic filaments in avian erythrocytes are composed of vimentin (Granger et al., 1982).

The question arises as to what vimentin binds on the nuclear and plasmalemmal surfaces. Georgatos and Marchesi (1985) have demonstrated that vimentin binds to the human erythrocyte membrane via a specific interaction with ankyrin, a component of the membrane skeleton. The relevance of this observation to the present discussion is equivocal for two reasons. First, since vimentin filaments are not present in the mature mammalian erythrocyte (see below), the physiological significance of vimentin-ankyrin binding is unknown. It is possible that vimentin instead binds to the plasma membrane via ankyrin in earlier stages of mammalian erythropoiesis, or alternatively may bind to some other membrane skeletal receptor, which may be present only during early stages of erythropoiesis. Second, non-mammalian ankyrin and vimentin may not be capable of binding to each other, and so the plasmalemmal association of intermediate filaments in nucleated erythrocytes would not be due to such an interaction. Direct binding studies using avian vimentin and avian membrane skeleton components will be needed to resolve this issue.

How do intermediate filaments attach to the nucleus? Ultrastructural examination of membrane-extracted fibroblasts have shown an attachment of intermediate filaments with the residual nuclear laminar material (Lehto et al., 1978). Interestingly, McKeon et al. (1986) have discovered a surprising sequence and structural homology between the nuclear lamins A and C and all other classes of intermediate filaments. Lamins A and C both contain the α-helical central rod domain characteristic of cytoplasmic intermediate filaments. These results suggest that the nuclear lamina is assembled via coiled-coiled oligomerization of the nuclear lamins, and furthermore that the nuclear lamins represent a sixth class of intermediate filament protein (McKeon et al., 1986). It will be interesting to see if nuclear lamins can form hetero-oligomers or heteropolymers with cytoplasmic intermediate filaments via coiled-coiled interactions. The structural homology between nuclear lamins and cytoplasmic intermediate filaments presents exciting new possible mechanisms for the attachment of intermediate filaments to nuclei.

As an approach to understanding the developmental and tissue-specific regulation of intermediate filaments, as well as the functional consequences of their expression, I have chosen erythropoiesis as a model system in which to study the expression of the vimentin gene. In Chapter 2 I present data demonstrating the isolation and identification of cloned DNAs specific for chicken vimentin, and an analysis of vimentin mRNAs in different chicken tissues and cells. Vimentin mRNA accumulates to high levels in chicken definitive erythroid cells, and this accumulation appears to underlie the synthesis and accumulation of vimentin filaments (Granger et al., 1982; Blikstad and Lazarides, 1983). In contrast to the avian erythrocyte, the anucleate mammalian erythrocyte contains no intermediate filaments. Immunofluorescence microscopy studies have shown that vimentin

expression is lost during the erythroblastic stages of human erythropoiesis in vivo (Dellagi et al., 1983). In Chapter 3 (Ngai et al., 1984) I demonstrate that vimentin expression is diminished upon chemically-induced differentiation of murine erythroleukemia (MEL) cells in vitro, and that the observed loss of vimentin filaments is due to a rapid decrease in vimentin mRNA levels. Differentiating MEL cells therefore provide an excellent model system in which to study vimentin gene regulation during cellular differentiation. The striking contrast in vimentin expression in avian and mammalian erythropoiesis led to a speculation that the loss of vimentin filaments during mammalian erythroid terminal differentiation is necessary to facilitate enucleation (Ngai et al., 1984 [Chapter 3]). What is the molecular basis for the divergent regulation of vimentin expression in avian and mammalian erythropoiesis? Have the factors governing vimentin gene regulation during erythropoiesis changed since the divergence of birds and mammals, or alternatively, have vimentin sequences themselves diverged, rendering the present-day patterns of erythropoietic vimentin expression? In Chapter 4 I have addressed these questions by studying the behavior of transfected vimentin genes in differentiating MEL cells. Based on these results, I present a mechanism to explain the divergent regulation of vimentin in avian and mammalian erythropoiesis.

Protein 4.1 and the Erythrocyte Membrane Skeleton

A mesh-like protein network lines the inner surface of the mammalian erythrocyte plasma membrane, and is believed to confer upon this membrane its properties of strength and elasticity, and to influence the lateral mobility of membrane proteins (reviewed by Branton et al., 1981, Cohen, 1983, and Marchesi, 1985). The major proteins of this network, termed the membrane skeleton, are α-

and β-spectrin, which form an $(\alpha,\beta)_2$ tetramer and complex with actin to form a two-dimensional protein network. This spectrin-actin-based complex is bound to the plasma membrane primarily via a high affinity interaction with ankyrin, which in turn is bound to a subset of transmembrane anion transporter molecules (Bennett and Stenbuck, 1979a,b, 1980; Luna et al., 1979; Yu and Goodman, 1979; Hargreaves et al., 1980). Protein 4.1 facilitates the interaction of spectrin and actin by forming a ternary complex with these two components (Ungewickell et al., 1979; Fowler and Taylor, 1980; Cohen and Korsgren, 1980; Ohanian et al., 1984). Protein 4.1 also has been demonstrated to interact directly with two intrinsic membrane proteins, glycophorin (Anderson and Lovrien, 1984) and anion transporter (Pasternack et al., 1985), as well as with membrane phospholipid (Sato and Ohnishi, 1983). Protein 4.1 therefore plays an important role in the maintenance of the membrane skeleton.

The major membrane skeletal components of the mammalian erythrocyte have been identified in chicken erythrocytes (Chan, 1977; Weise and Chan, 1978; Repasky et al., 1982; Jay, 1983; Granger and Lazarides, 1984; Nelson and Lazarides, 1984). Protein 4.1 exists as a set of structurally related polypeptides in avian erythroid cells (Granger and Lazarides, 1984). In chicken erythrocytes, there are seven major protein 4.1 variants of 77, 87, 100, 115, 150, 160, and 175 kDa, with the 100 and 115 kDa polypeptides found in highest abundances (Granger and Lazarides, 1984, 1985). In immature mitotic erythroblasts, the 77 and 87 kDa protein 4.1 variants predominate, and as the cells undergo terminal differentiation, the 100 and 115 kDa polypeptides accumulate to higher levels (Granger and Lazarides, 1985). Hence, the expression of multiple protein 4.1 variants is developmentally regulated during chicken erythropoiesis. A kinetic analysis of protein 4.1 synthesis and assembly onto the membrane skeleton

indicates that protein 4.1 variants do not arise by posttranslational processing pathways (Granger and Lazarides, 1984; Staufenbiel and Lazarides, 1986). The complexity of protein 4.1 expression in chicken erythrocytes raises the question as to how these multiple variants are generated. Several lines of evidence have given preliminary indications that multiple protein 4.1 polypeptides are the product of a single gene. First, turkey erythrocytes contain protein 4.1 variants similar to those found in chickens, but each variant is consistently ~3 kDa smaller (Granger and Lazarides, 1984). It would be difficult to reconcile these consistent interspecies differences with the presence of multiple protein 4.1-encoding genes. Second, a human patient presenting a mutant form of erythroid protein 4.1 has been described, wherein the mutation yielded an 8.5 kDa truncation of both of the proteins which comprise the normal protein 4.1 doublet in humans (Alloisio et al., 1982). A single genetic locus apparently was responsible for this mutation (Alloisio et al., 1981).

In Chapter 5 (Ngai et al., 1987) I present data showing the isolation and characterization of cDNA clones specific for chicken erythroid protein 4.1. I have used these cDNAs as probes in a preliminary investigation of the mechanisms governing the production of multiple protein 4.1 variants.

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CHAPTER 2:

Tissue-Specific Expression of Two mRNA Species Transcribed from a Single Vimentin Gene

(Appeared in *Cell*, Vol. 35, pp. 411-420, 1983)

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Tissue-Specific Expression of Two mRNA Species Transcribed from a Single Vimentin Gene

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Summary

We have isolated chicken cDNA and genomic clones for the intermediate filament subunit vimentin and show that the gene for this protein, which exists in a single copy in the haploid chicken genome, is transcribed into two mature mRNA species with approximate lengths of 2.0 and 2.3 kb. We have found cell-specific regulation in the expression of these two mRNAs; whereas both mRNA species are present in muscle cells, fibroblasts, spinal cord and lens, erythroid cells from 10- and 15-day-old chicken embryos express predominantly the lower molecular weight RNA. The difference between these two mRNAs is due to different lengths of their 3′ untranslated regions, suggesting that the cell-specific regulation of their expression occurs either by specific termination of transcription or by differential post-transcriptional processing of the 3′ untranslated region. Additionally, a remarkable induction (40- to 50-fold) in the abundance of the vimentin mRNA is observed in erythroid cells as chicken development proceeds from 4 to 15 days, which suggests that the level of expression of vimentin during erythroid development is regulated at the transcriptional level.

Introduction

The expression of intermediate filament subunits represents an attractive model system for studying regulation of gene expression during development and differentiation in higher eukaryotic organisms. The five major classes of subunits identified to date share a number of structural and biochemical similarities and generally exhibit specificity of expression in certain cell types: keratins are expressed in epithelial cells, neurofilaments in neurons, glial filaments in glial cells and desmin in muscle cells. A major exception is the intermediate filament subunit vimentin; even though it is the predominant subunit expressed in certain mesenchymally-derived cell types, its expression is more generalized (for reviews see Lazarides, 1980, 1982). Certain terminally differentiating cells substitute a cell-type specific subunit for vimentin upon their commitment to differentiate (e.g. neurons; Tapscott et al., 1981), while in others expression of vimentin persists through the fully differentiated state. In the latter case, vimentin can be the sole subunit expressed which is best exemplified in chicken erythrocytes (Granger et al., 1982). Alternatively, vimentin is coexpressed with a cell type-specific subunit [e.g. chicken skeletal muscle (Granger and Lazarides, 1979), certain mouse retinal neurons (Dräger, 1983), and chicken and rat astrocytes (Tapscott et al., 1981; Yen and Fields, 1981)]. The modulation of the ratio of vimentin and the cell-type specific subunit may be a function of their differential synthesis and turnovers. Whether indeed this heterogeneity in the composition of certain intermediate filament systems is regulated by the differential abundance in the mRNAs of the two subunits is presently unknown.

In addition, intermediate filaments exhibit a rearrangement in their cytoplasmic distribution in cultured myogenic cells, where a cytoplasmic filament-type distribution is replaced with a sarcomere Z-line association (Gard and Lazarides, 1980). This rearrangement is presumed to reflect a functional property of these filaments in muscle morphogenesis (Granger and Lazarides, 1978; Gard and Lazarides, 1980; Lazarides, 1980). How this morphogenetic redistribution is regulated during the terminal stages of muscle differentiation is also undetermined.

To probe the above observations and to further define the modes of expression of the various intermediate filament subunits, we have examined the expression of vimentin mRNA in a variety of tissues and in different stages of differentiation of certain cell types by the use of recombinant clones of chicken vimentin. We show that the vimentin gene, which is represented only once in the chicken genome (Zehner and Paterson, 1983), spans at least 8 kb of DNA and is transcribed into two distinct mRNA species which exhibit cell specificity in their expression. The difference in the lengths of the two mRNAs is primarily due to different lengths of their 3′ untranslated regions, suggesting that the expression of the two mRNAs is regulated by cell-type specific termination of transcription or by differential post-transcriptional processing (but not splicing) of the 3′ untranslated region. Furthermore, we show that the accumulation of vimentin filaments during terminal differentiation of erythrocytes is regulated mainly at the transcriptional level or by RNA stabilization.

Results

Isolation and Characterization of Vimentin Gene Recombinant Clones

Recombinant DNA clones containing vimentin gene sequences were isolated by a combination of two strategies carried out independently. First, we have used the following mixed 16-fold degenerate, 14-nucleotide-long synthetic probe,

```
3′  T T G C A G T T C T A C C G  5′
        A     C     T
              A
              T
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deduced from the amino acid sequence of chicken vimentin (Geisler and Weber, 1981),

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61                  64
...Asn-Val-Lys-Met-Ala
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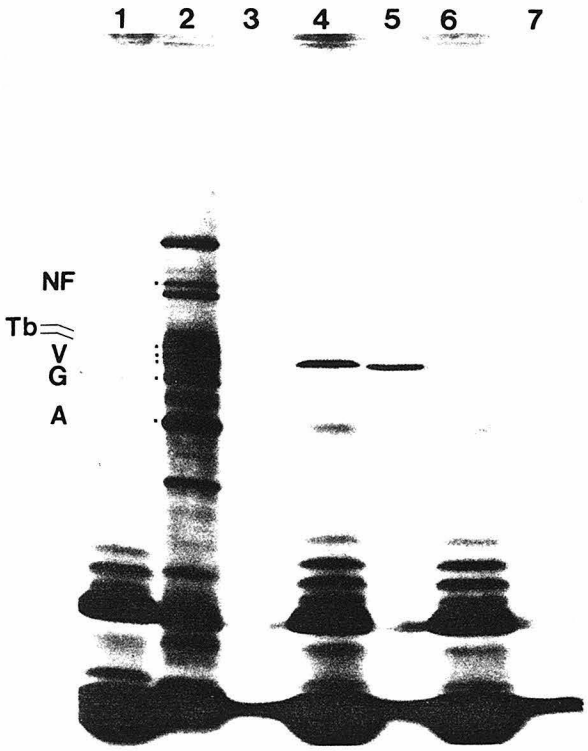

Figure 1. Positive Hybrid-Selected Translation of Vimentin mRNA

Hybrid-selected translations were performed as described in Experimental Procedures, and the resulting translation products and immunoprecipitates were resolved on a 12.5% SDS-polyacrylamide gel and detected by fluorography. For a given *in vitro* translation reaction, an equivalent aliquot of non-precipitated lysate was loaded as was used for immunoprecipitation. Lane 1, endogenous activity of lysate; lane 2, translation products from unfractionated 2-week-old chicken spinal cord poly(A)$^+$ RNA; lane 3, the corresponding immunoprecipitation with anti-vimentin; lane 4, translation products selected by p5C5 plasmid DNA and lane 5, immunoprecipitation with anti-vimentin; lane 6, translation products selected by pBR322 and lane 7, immunoprecipitation with anti-vimentin. Note that the amount of vimentin synthesized from spinal cord poly(A)$^+$ RNA is just detectable at these loadings. NF, 70 kd neurofilament subunit, Tb, tubulins, V, vimentin, G, glial fibrillary acidic protein, A, actin.

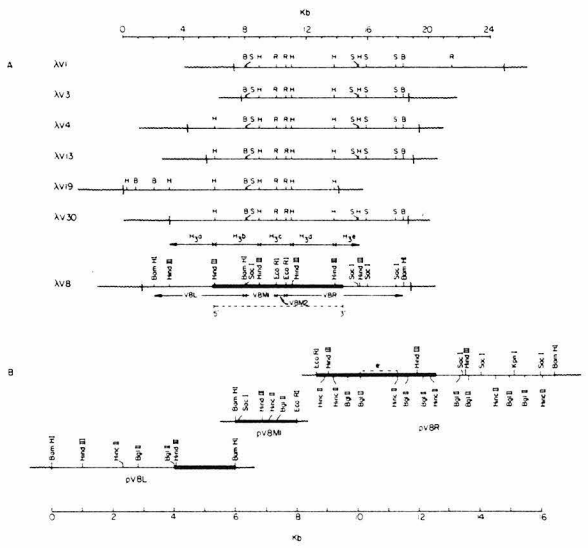

Figure 2. Restriction Map Analysis of the Vimentin Gene

(A) Comparison of the vimentin gene isolates. Representative phage clones of the seven insert size-distinct classes containing the following number of isolates per group: λV1, 1; λV3, 5; λV4, 4; λV8, 6; λV12, 1; λV19, 6; λV30, 1. (B) Detailed restriction maps of three pBR322 subclones containing the vimentin gene. EcoR1-Bam HI and Bam HI fragments of λV8 were subcloned in pBR322. The origins of the subclones are noted below the corresponding λV8 fragments. The thick line designates the vimentin gene. Flanking sequences are shown by thin straight lines. The direction of transcription found by hybridization of the 3′ synthetic probe to DNA blots of different restriction fragments is also indicated and the position where the synthetic probe hybridizes is noted as well (–•–). The EcoRI fragment V8M2 is not represented in these three subclones. B, Bam HI; H, Hind III; R, EcoR1; S, Sac I.

to screen two genomic equivalents of a partial HaeIII-AluI chicken genomic DNA Charon 4A library (Dodgson et al., 1979). This sequence is 76 amino acids from the carboxyl terminus. Only three clearly positive recombinant DNA clones were obtained; each had a distinct restriction map and did not cross-hybridize with the others.

Simultaneously, we isolated a vimentin cDNA clone from a chicken spinal cord cDNA library in the following manner. The cDNA library first was screened with ^{32}P-labeled cDNAs synthesized from size-fractionated chicken spinal cord poly(A)$^+$ RNA, as described in Experimental Procedures. Clones which hybridized preferentially with probes generated from a vimentin-enriched fraction were subjected to further analysis by positive hybrid-selected translation. Figure 1, lane 4 shows the *in vitro* translation product from RNA which bound specifically to one recombinant cDNA plasmid, p5C5. The identity of this translation product as vimentin was confirmed by immunoprecipitation with an anti-chicken vimentin antiserum (lane 5), and by two-dimensional electrophoresis (data not shown). Lanes 6 and 7 represent translation products from RNA selected by pBR322 and the corresponding immunoprecipitation with anti-vimentin, respectively; the amount of vimentin mRNA bound to pBR322 under these conditions is below the detection limit of our procedures. From these experiments we conclude that p5C5 is a vimentin cDNA plasmid.

By hybridizing p5C5 with the three genomic clones selected by the synthetic oligonucleotide probe, we established that only one of them contains vimentin gene sequences. Screening four more genomic equivalents of the same library with p5C5 yielded 24 independent vimentin gene-containing phages. Restriction analysis of these vimentin isolates showed that they all share several identical restriction fragments and represent seven insert size-distinct classes as shown in Figure 2A [multiple isolates of most recombinants were obtained, probably due to plate growth amplification (Dodgson et al., 1979)]. Further restriction analysis of representative clones suggested that they all contain overlapping fragments of the same vimentin gene. Genomic DNA blot analysis as well as genomic reconstitution experiments demonstrated the existence of a single vimentin gene in the chicken genome (data not shown), confirming the data of Zehner and Paterson (1983), reported while this work was in progress.

The orientation of transcription within these segments was established by hybridization of the 3′ end-specific ^{32}P-labeled synthetic oligonucleotide probe to DNA blots of restricted vimentin genomic clones (data not shown). The approximate position of the synthetic sequence is shown in Figure 2B. This position is at least 400 bp away from the one predicted both from the location of the peptide from which this synthetic probe was deduced and from further data of the map of the gene (see below), implying the existence of an intron close to the end of the translated region of the vimentin gene. On the other hand, hybridization of the p5C5 clone to similar DNA blots of restricted vimentin phage DNA indicates that the cDNA insert corresponds to the 5′ end of the vimentin gene and that the gene extends more than 2 kb upstream from the EcoRI fragment V8M2 as shown in Figure 2A. Furthermore, the p5C5 hybridization pattern reveals that this area of the vimentin gene is interrupted by another intron containing both the 2 kb V8M1 and the 0.65 kb V8M2 fragments (Fig. 2A), as p5C5 hybridizes to the gene both left and right of these segments, but not to them (data not shown).

Since the EcoRI sites are near the middle of the gene, we considered the λV8 clone, shown in Figure 2A, as the most representative, and subcloned it in pBR322. The chimeric plasmids were constructed by ligation of EcoRI-BamHI-, BamHI-, and HindIII-digested fragments of the genomic λV8 clone with pBR322 plasmids, and were used to transform *E. coli* HB101 (see Experimental Procedures). In this way we obtained subclones of all possible EcoRI-BamHI, BamHI, and HindIII fragments of the λV8 genomic clone. A detailed restriction map of three subclones, pV8L, pV8M1, and pV8R, corresponding to most of the λV8 insert is shown in Figure 2B. The 5′- and 3′-boundaries of the vimentin gene were approximated by hybridization of different pV8 restriction fragments to RNA blots (data not shown). In the case of the 5′ end, the ^{32}P-labeled left end of the H_3b fragment was protected by RNA from S1 nuclease (data not shown), whereas hybridization of RNA blots with sequences 5′ to the H_3b fragment was negative. Assuming that these sequences are not within an intron, the initiation of transcription of the vimentin gene must be located within ~50 bp 5′ to the left HindIII site of the H_3b fragment as shown in Figure 2. Our approximation of the 5′ end of the vimentin gene does not agree with that recently reported by Zehner and Paterson (1983); these authors located the 5′ end of the gene near one of the internal EcoRI sites. However, the position of the 3′ end border, as shown in Figure 2, agrees with the one predicted by the sequence data of Zehner and Paterson (1983).

Two Different Size mRNAs are Transcribed from the Single Vimentin Gene with Cell-Specificity in their Expression

To investigate the expression of the vimentin mRNA in different cell types, during myogenesis and during erythroid development, the following experiments were carried out. Total and/or polyadenylated RNA from gizzard, skeletal muscle, spinal cord, lens fibers, 10-day-old embryos, chicken embryo fibroblasts, myogenic cultures at different stages of differentiation, and erythroid cells from different stages of chicken embryonic development were fractionated by electrophoresis in agarose gels containing formaldehyde and transferred to nitrocellulose paper. The immobilized RNA was then hybridized with a nick-translated probe synthesized either from the entire pV8R (Figure 2) or portions therefrom. The results of such experiments are shown in Figures 3 and 4. Two distinct size classes of vimentin mRNA can be identified, with molecular lengths of 2.0 and 2.3 kb, as shown by Zehner and Paterson (1983) for myogenic cells. Both mRNA species are present in muscle cells, fibroblasts, spinal cord and lens at similar concentrations. However, from Figure 3A it is evident that with the exception of skeletal muscle, in the other tissues the 2.3 kb RNA is 1.5–2 times more prevalent than the 2.0 kb species. Both mRNA species were detected in 14-day embryonic muscle polysomal poly(A)$^+$ RNA (data not shown), indicating that both are mature mRNAs.

Surprisingly, erythroid cells obtained from 10- and 15-day-old embryos express predominantly the lower molecular weight vimentin mRNA (Figure 3 A; Figure 4 A and B); comparatively very low amounts of the 2.3 kb RNA species are present in both cases. At the same level of exposure, no vimentin RNA band could be detected in total or poly(A)$^+$ RNA from 4-day embryonic erythroid cells (Figure 4, A and B). Prolonged exposure of this blot (~7X) reveals the presence of very low levels of vimentin mRNA and a lower difference in the intensities of the two mRNA bands. The increase in vimentin mRNA from 4- to 10-day cells is approximately 30-fold, whereas there is a smaller increase (less than two times) from 10- to 15-day cells. Comparison of total and poly(A)$^+$ erythrocyte RNA blots (Figure 4, A and B) demonstrates a smaller increase in the prevalence of the 2.0 kb RNA species in the poly(A)$^+$ RNA population from 10- to 15-day cells. This might arise from a higher degree of vimentin RNA polyadenylation in the 10-day as compared to the 15-day erythroid cells.

Two main conclusions emerge from the observations described above. First, there is specific expression of predominantly the lower molecular weight vimentin RNA species in erythroid cells from 10- and 15-day chicken embryos (Figures 3 and 4). Second, there is a remarkable (40- to 50-fold) increase in the level of expression of this 2.0 kb RNA in erythroid cells as development proceeds from the 4th to the 15th day (Figure 4).

Figure 3B shows RNA blot analysis of cultured myogenic cells at 6, 12, and 64 hr after plating. There is an almost equimolar representation of the two RNA species throughout myogenesis with a slight increase in the relative concentration of the 2.0 kb RNA as differentiation proceeds.

With regard to the level of vimentin expression in the different tissues or cells tested, cultured myogenic cells and chicken embryo fibroblasts contain the highest levels of vimentin RNA. Additionally, it is evident that the amount of vimentin RNA in gizzard is ~50-fold lower than in 10-day-old total embryo and 10-fold lower than in skeletal muscle (Figure 3).

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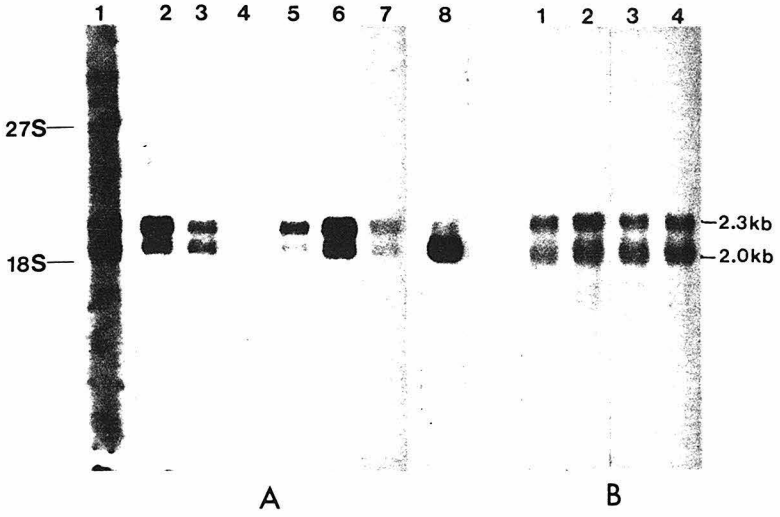

Figure 3. Expression of Vimentin RNA in Different Chicken Cell and Tissue Types and During Myogenesis In Vitro

(A) Total polyadenylated RNA isolated from different sources was fractionated by electrophoresis on 1.3% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose filters and hybridized to nick-translated probe synthesized from either the entire pV8R or part of it. Lane 2, 10-day-old embryo (1.5 μg); lane 3, 1-week-old skeletal muscle, (3 μg); lane 4, 1-week-old gizzard smooth muscle, (5 μg); lane 5, 2-week-old spinal cord, (3 μg); lane 6, 1-week-old lens, (3 μg); lane 8, 15-day-old embryonic erythrocytes, (3 μg). The quantities of the RNA used were intentionally unequal due to high prevalence differences. Lane 7 is a three-fold longer exposure of the gizzard lane (4). Lane 1 shows a blot similar to lane 2 hybridized with the synthetic oligonucleotide probe as described in Experimental Procedures.

(B) Similar RNA blots carried out as (A) using 10 μg total RNA from different stages of cultured myogenic cells 6 hr (1), 12 h (2), and 64 h after plating (3), and chicken embryo fibroblasts (4) hybridized to p5C5. The positions of 27S and 18S rRNA were obtained by ethidium bromide staining of 5 μg total chicken muscle RNA from adjacent lanes.

The Two Size Classes of Vimentin mRNAs Differ in the Lengths of Their 3′ Untranslated Regions

The data presented so far demonstrate that the single vimentin gene is transcribed into two different size classes of mRNA with cell-specific expression. Unmodified chicken vimentin synthesized *in vivo* migrates as one major 52,000 dalton protein in both one- and two-dimensional gel electrophoresis (see Lazarides, 1980, 1982). This is also the result of our hybridization selection experiments where *in vitro* translation of the selected RNA and immunoprecipitation of the translated products give only one size protein (Figure 1), thus suggesting that the ~300 nucleotide difference between the two vimentin mRNAs is not due to large differences in protein coding regions but arises predominantly from differences in untranslated sequences. In an attempt to find the segment of the gene that encodes only one of the two mRNAs we hybridized RNA blots to a series of probes from different regions near the 5′ end and 3′ end of the vimentin gene. All probes hybridized to both RNAs (e.g. see Figure 5, fragment HindIII-HincII), except the HincII-HindIII fragment from the 3′ end of the gene, shown in Figure 5, which hybridized only to the longer mRNA species. The preferential expression of the lower molecular weight mRNA in 15-day erythrocytes is shown once more by this experiment. The DNA probe containing the rest of the 3′ sequences of the pV8R insert (Figure 5) did not hybridize to either mRNA. These data demonstrate that the difference between the two size classes of vimentin mRNA lies mainly in the lengths of their 3′ untranslated regions and is not due to differential splic-

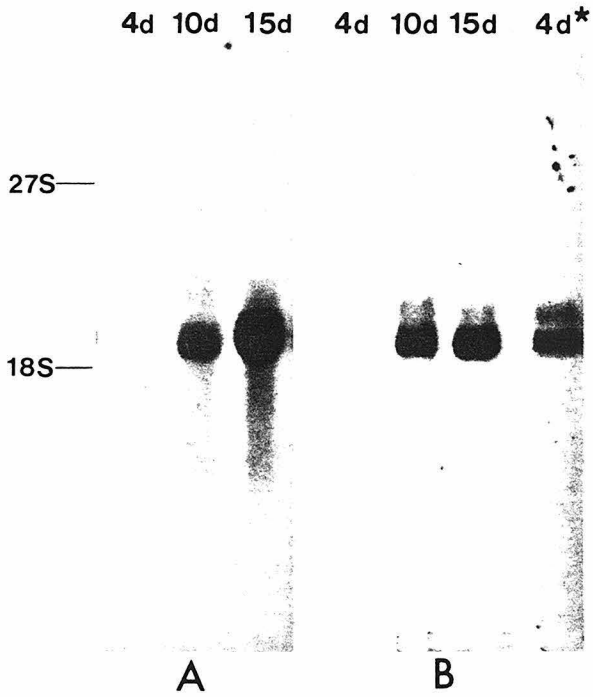

Figure 4. Induction in the Expression of Vimentin mRNA during Erythroid Development

RNA blots similar to those described in Figure 3 were performed with either 10 μg erythrocyte total RNA (A) or 3 μg erythrocyte poly(A)$^+$ RNA (B) obtained from 4-day-old (4 d), 10-day-old (10 d) and 15-day-old (15 d) embryos, as designated. 4d* is a 7-fold longer exposure of lane 4d. The positions of 27S and 18S rRNA were obtained as described in Figure 3.

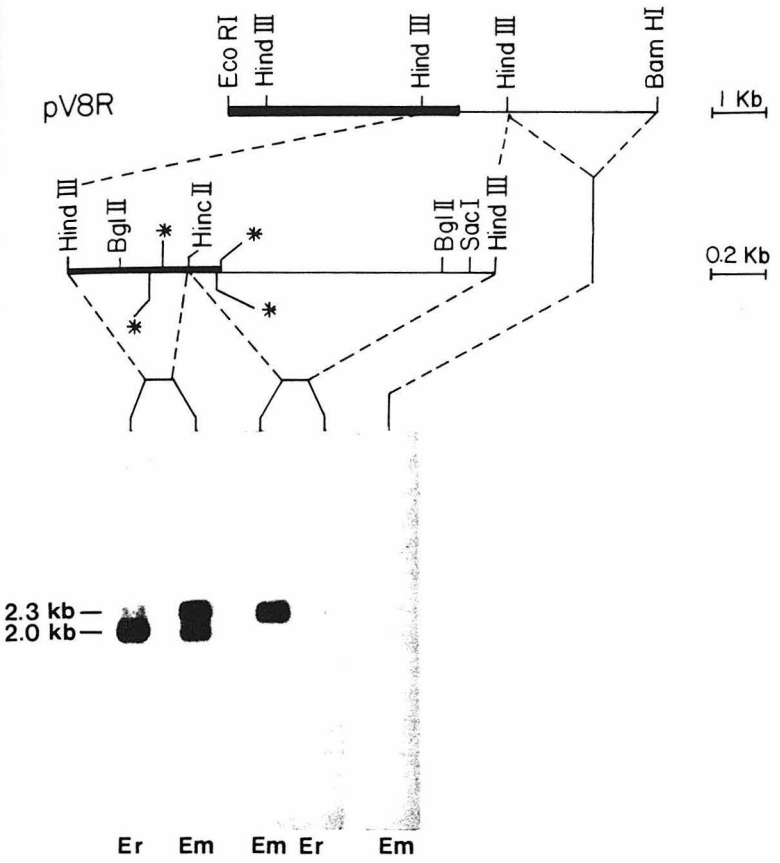

Figure 5. Localization of the 3′ Untranslated Sequence Responsible for the Size Differences of the Two Vimentin mRNAs

The lower part of the figure shows blots of RNA from 10-day-old embryos and 15-day erythrocytes hybridized to different fragments of the 3′ end and beyond the 3′ end of the vimentin gene, as designated. The upper portion of the figure shows the restriction map of the 3′ end of the gene and the fragments used for these hybridizations. The 450 bp Hind III- Hinc II fragment and the 1150 bp Hinc II-Hind III fragments were isolated from low-melt agarose gels, from the 1.6 kb Hind III fragment H_3e and after labeling by nick-translation were hybridied to RNA blots. The 2.8 kb Hind III-Bam HI fragment beyond the 3′ end was isolated from the pV8R subclone and labeled as described above. The asterisks (*) show the two polyadenylation site sets derived from the sequence data of the 3′ end of the gene (Zehner and Paterson, 1983).

ing at the 3′ end of the gene. While this work was in progress, Zehner and Paterson (1983) reported the sequence of the 3′ untranslated region of the vimentin gene which revealed two sets of tandemly repeated putative polyadenylation sites approximately 250 nucleotides apart. The HincII site we have used to generate the 3′ segment of the gene that hybridized only to the larger RNA species is located between these tandem polyadenylation sites, 90 bp 3′ to the second site of the first set (Figure 5). We have searched further for any possible differences in the transcription of the 5′ end of the vimentin gene by S1 nuclease mapping using both embryonic and erythrocyte RNA; no differences could be detected at our level of resolution (~50 bp) (data not shown).

Discussion

Transcription of the Single Vimentin Gene into Two mRNAs with Cell-Specificity in Their Expression

We report here the isolation, characterization, and pattern of expression of the chicken vimentin gene. Restriction map analysis and genomic DNA blot experiments confirmed the observation that vimentin is present as a single copy in the haploid chicken genome (Zehner and Paterson, 1983). Experiments by Dodemont et al. (1982) also have suggested the presence of a single vimentin gene in the hamster genome. We show that the single chicken vimentin gene is transcribed into two different size mRNA species of 2.0 kb and 2.3 kb with cell-specific regulation in their expression. Whereas both mRNA species are represented in chicken muscle cells, fibroblasts, spinal cord, and lens, predominantly the lower molecular weight mRNA is expressed in erythroid cells obtained from 10- and 15-day-old chicken embryos. Erythroid cells from 4-day-old embryos, however, express both mRNAs in comparatively low amounts and with a much smaller difference in the relative abundance of the two species.

Blood cells from 4-day-old embryos are a mixture of early and late polychromatophilic primitive erythroblasts, whereas cells from 10-day-old embryos are ~25% primitive cells, ~35% mid to late polychromatophilic definitive erythroblasts, and ~35% mature definitive erythrocytes; approximately 60–70% of the cells from 15-day-old embryos are mature definitive erythrocytes, with the remainder being late polychromatophilic definitive erythroblasts (Bruns and Ingram, 1973). We do not know which of these cells synthesize vimentin or the relative quantities of vimentin produced by each cell type. Nevertheless, since primitive and definitive erythrocytes arise from different erythroid cell lines, the induction of accumulation of the 2.0 kb vimentin mRNA found between 4 and 10 days of

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development should be considered as lineage-specific. Furthermore, the low abundance 2.3 kb vimentin mRNA in 10- and 15-day-old cells most likely does not derive from primitive series cells, as these cells comprise only a small percentage of the 10-day population, and are virtually absent in 15-day-old embryo blood.

The tremendous induction in the 2.0 kb vimentin mRNA expression from 4- to 15-day embryonic erythroid cells underlies similar changes at the protein level (Blikstad and Lazarides, unpublished results), strongly suggesting primarily transcriptional and/or posttranscriptional, but not translational control in the expression of vimentin during erythroid development. Transcriptional and/or posttranscriptional control in the level of vimentin expression may apply to the rest of the cell and tissue types studied as well, as comparative abundances in vimentin mRNA generally correlate with the observed respective protein levels. This is best exemplified in the case of gizzard, where the low levels of vimentin detected (Granger and Lazarides, 1980), reflect similarly low levels of vimentin RNA.

The Two Vimentin mRNAs have Unequal Lengths in Their 3′ Untranslated Regions

By hybridizing RNA blots from both erythrocytes and embryos to different fragments of the vimentin gene (Figure 5), we have shown that the difference between the two size classes of vimentin mRNA, transcribed from a single gene, is due to different lengths of their 3′ untranslated regions. Hence, it is clear that during erythroid development erythrocytes regulate vimentin expression either by differential termination of transcription or by differential processing of the 3′ end. At present we have no evidence supporting either of these two possibilities. An answer to this question will require short time periods of labeling and detection of the vimentin primary transcripts. *In vitro* transcription studies have shown continuous transcription of the mouse β-major globin gene extending 1400 nt beyond the poly(A) addition site (Hofer and Darnell, 1981), suggesting the necessity of an endonucleolytic cleavage of a primary transcript followed by polyadenylation in the process of creating the 3′ end of this mRNA, as has been demonstrated for the adenovirus and SV40 transcription units studied (Nevins and Darnell, 1978; Ford and Hsu, 1978; Lai et al., 1978; Faser et al., 1979; Nevins et al., 1980). We therefore cannot exclude a precursor-product relationship in which the 2.3 kb mRNA is cleaved and secondarily polyadenylated, thus giving rise to the 2.0 kb mRNA. The size heterogeneity in the 3′ untranslated regions of mRNAs derived from a single vimentin gene resembles those reported for the mouse dihydrofolate reductase gene which codes for four mRNAs (Setzer et al., 1980), as well as the mouse β_2-microglobulin (Parnes and Robinson, 1983), and α-amylase genes (Tosi et al., 1981), both of which code for two mRNAs. Since the biological functions of 3′ untranslated regions is unknown, the significance of variations in their lengths is unclear. The important implication of our data, however, is directly related to this question. The tissue-specific differences in the relative distribution of the two size classes of vimentin mRNA imply that the presence and usage of multiple poly(A) addition sites are biologically significant. It is possible that the differences in the 3′ untranslated region somehow affect the subcellular compartmentation of the mRNA (and consequently the fate of the translated protein), the relative stabilities of the two mRNAs, or both.

Functional Implications of the Induction of Vimentin mRNA Accumulation During Erythroid Development

Preliminary studies from this laboratory have shown that the primitive series of erythroid cells in the chicken contain and synthesize very low levels of vimentin (Blikstad and Lazarides, unpublished observations). However, there is a dramatic increase in the synthesis and accumulation of vimentin when the definitive series of erythroid cells appears in the blood of the animal (Blikstad and Lazarides, 1983), with a concomitant appearance of clearly identifiable intermediate filaments (Granger and Lazarides, 1982). The observed increase in the accumulation of vimentin mRNA in the definitive series erythroid cells (Figure 4) argues that this accumulation of vimentin is regulated either at the level of transcription or by RNA stabilization. How this induction in the transcription or accumulation of vimentin mRNA is regulated during the switch of the organism from the production of the primitive series to the production of the definitive series of cells is unknown. One possibility is that the level of induction is influenced by hemopoietic microenvironments at the sites of production of the definitive series cells. Alternatively, the level of vimentin expression may be a function of stem cell heritage. Nevertheless, the large induction in vimentin synthesis suggests that vimentin filaments are an essential part of the terminal differentiation program of these cells. The increased accumulation of vimentin in definitive erythroid cells should be contrasted and compared with the expression of intermediate filaments in other terminally differentiating cells such as neurons and muscle cells. In these cases the cells substitute the pre-existing vimentin filaments with a cell type-specific intermediate filament subunit, either completely, as in the case of the neurofilament subunit in neuronal differentiation (Tapscott et al., 1981), or partially as in the case of desmin in muscle differentiation (Gard and Lazarides, 1980). Since, at least in the case of erythroid cells, regulation of vimentin expression occurs predominantly at the level of mRNA abundance, such partial or complete changes in the composition of intermediate filaments during terminal differentiation in other cell types also may be regulated at the mRNA level.

Implications for the Regulation of Vimentin Distribution During Myogenesis

Developing myogenic cells exhibit a striking redistribution of vimentin- and desmin-containing filaments which occurs during the terminal phases of differentiation (Kelly, 1969; Bennett et al., 1979; Gard and Lazarides, 1980). In the

initial stages of myogenesis vimentin and desmin assemble into cytoplasmic intermediate filaments. However, after the assembly of functional, contractile sarcomeres, vimentin and desmin begin to associate with the myofibril Z-discs (Gard and Lazarides, 1980), where they surround and link them laterally to each other and to other cytoplasmic organelles (Granger and Lazarides, 1978, 1979). Since vimentin filaments exhibit slow turnover *in vivo* (McTavish et al., 1983; Blikstad and Lazarides, 1983), the redistribution of vimentin could be regulated at the transcriptional level by the activation of a Z-disc specific vimentin gene, posttranslationally by a specific chemical modification of vimentin, and/or by the elaboration of a specific Z-disc vimentin receptor which induces these filaments to rearrange and bind to the peripheries of Z-discs. The first possibility is unlikely, as we and others (Zehner and Paterson, 1983) have shown that vimentin is present as a single gene in the chicken genome, and moreover, the major difference between the two mRNA species appears to lie in the 3′-untranslated regions. This is in accordance with previous studies from this laboratory which show that the vimentin protein exhibits no detectable changes in its electrophoretic mobility or isoelectric point before and after transition to the Z-line (Gard and Lazarides, 1980). However, we have observed that vimentin (and desmin) become susceptible to changes in their phosphorylation by cAMP-dependent protein kinases at a time which coincides with the onset of this redistribution to the Z-line (Gard and Lazarides, 1982). Collectively these observations suggest that the redistribution of vimentin filaments during the terminal phases of myofibril assembly is regulated primarily posttranslationally and/or by the appearance of a specific Z-disc vimentin receptor(s).

Experimental Procedures

Isolation of RNA

Gizzards, skeletal muscle, and lens fibers were isolated from 1-week-old chicks and spinal cords from 2-week-old chicks. Chicken embryo erythroid cells were isolated from 4-, 10-, and 15-day-old embryos as previously described (Granger et al., 1982). Cultures of embryonic myogenic cells and chicken embryo fibroblasts were prepared from 10- to 11-day-old chicken embryos as previously described (Gard and Lazarides, 1980). Total RNA was isolated essentially according to the method described by Chirgwin et al. (1979). Pulverized frozen tissue or cell pellets were homogenized in 5 M guanidinium thiocyanate, 50 mM Tris, pH 7.5, 50 mM EDTA, 5% 2-mercaptoethanol, and 3% sodium lauryl sarcosine using a Dounce homogenizer. The homogenates (~30 ml) were layered over 8 ml 5.7 M CsCl cushions and centrifuged at 39,000 rpm for 20 hr in a Beckman 60 Ti rotor. The pellets were dissolved in water and precipitated with ethanol twice. Polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography essentially as described by Aviv and Leder (1972).

Construction and Screening of the Spinal Cord cDNA Library

Synthesis of cDNA from 2-week-old chicken spinal cord poly(A)$^+$ RNA was essentially performed as outlined by Efstratiadis and Villa-Komaroff (1979) using oligo(dT)$_{12-18}$ as a primer. Second strand synthesis was carried out with DNA polymerase I. The resulting double stranded cDNA was digested with nuclease S1 and chromatographed on a Biogel A150 m column. Leading fractions were pooled, yielding cDNAs with lengths of 0.5 to >1.6 kb. This cDNA was homopolymerically tailed with dCTP by incubation with terminal transferase (Rowekamp and Firtel, 1980); PstI-digested, gel purified pBR322 was similarly reacted with dGTP. Approximately equimolar amounts of dCMP-tailed cDNA and dGMP-tailed vector were annealed and used to transform *E. coli* HB101 (Cohen et al., 1972).

^{32}P-labeled cDNAs generated from size-fractionated spinal cord poly(A)$^+$ RNA were utilized as probes in a preliminary screening of the cDNA library. Poly(A)$^+$ RNA from spinal cords of 2-week-old chickens was electrophoresed in the presence of methylmercury hydroxide (Bailey and Davidson, 1976) on a preparative 1% low melting point agarose gel. The gel was soaked in 0.2 M potassium acetate (pH 5.0), 1 mM EDTA, 13 mM DTT, and then sliced repetitively perpendicular to the direction of electrophoresis. A volume of the above buffer approximately equal to that of each gel slice was added to each fraction, the gels were melted at 60°C, and RNA was purified by extraction with phenol (Weislander, 1979).

To determine the mRNA composition of each fraction, aliquots were used to direct protein synthesis in a nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976) containing ^{35}S-methionine. The resulting translation products were subjected to both one- and two-dimensional gel electrophoresis (Laemmli, 1970; O'Farrell, 1975; Hubbard and Lazarides, 1979); the gels were impregnated with Enhance (New England Nuclear), dried and exposed to Kodak XAR film.

Fractions which were determined by *in vitro* translation to be enriched in vimentin mRNA served as substrates for reverse transcriptase in the presence of α-^{32}P-labeled deoxynucleotides, using sheared denatured calf thymus DNA as primer (Taylor et al., 1976). Filters bound with DNA of colonies bearing recombinant plasmids were hybridized with ~2 × 10^6 cpm probe/ml for 48 hr at 42°C (Grustein and Hogness, 1975). To identify and reduce the number of non-vimentin positives, the library was counter-screened in a similar fashion using ^{32}P-labeled cDNAs synthesized from RNA fractions depleted of vimentin mRNA, as well as from gizzard poly(A)$^+$ RNA.

Positive Hybrid-Selected Translation

Complementary DNA clones which hybridized differentially with the vimentin-enriched ^{32}P-labeled cDNA probe were pooled in groups of up to 6 and further tested by positive hybrid-selected translation (Ricciardi et al., 1979). Twenty micrograms of EcoRI-restricted, denatured plasmid DNA, was spotted on nitrocellulose (Kafatos et al., 1979) with the aid of a Hybridot Manifold (Bethesda Research Laboratories). Filters were washed with 1 M NH_4OAc and 1X SSC (1X SSC is 150 mM NaCl, 15 mM Na-citrate), and baked at 70°C under vacuum. Prior to hybridization, filters were boiled in H_2O and prehybridized in 50% formamide, 0.1 M PIPES, 0.6 M NaCl, pH 6.4, 50 μg/ml poly(A) for 2 hr at 54°C. Nine micrograms of 2-week-old chicken spinal cord poly(A)$^+$ RNA was hybridized for 3 hr with each filter in 30 μl of the above buffer minus poly(A) at 54°C. Filters were then washed ten times with 1XSSC, 0.5% SDS at 65°C, twice with 10 mM Tris-HCl, 2 mM EDTA, pH 8.0 at room temperature, and once with 10 mM Tris-HCl, pH 7.5 at room temperature. Bound RNA was eluted by boiling the filters for 60 sec in 100 μl H_2O and quenching in a dry ice/ethanol bath. Three micrograms of carrier beef liver tRNAs and 10 μl 2 M NH_4OAc were added, and the samples were precipitated and washed twice with 70% ethanol. The RNA pellets were then used to direct protein synthesis in a rabbit reticulocyte lysate as described in the previous section. Immunoprecipitations were carried out using a high-titer rabbit anti-chicken vimentin antiserum (Granger and Lazarides, 1979) essentially according to method B of Blikstad et al. (1983), except that all immunoprecipitation buffers contained 0.25% 2-mercaptoethanol to reduce background. Immunoprecipitates as well as non-precipitated samples were electrophoresed on 12.5% SDS-polyacrylamide gels, impregnated with Enhance and subjected to fluorography.

Synthesis of a Mixed Oligonucleotide Probe

The mixed oligonucleotide probe

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3′-T T G C A G T T C T A C C G-5′,
      A     C     T
            A
            T
```

derived from the amino acid sequence, Asn-Val-Lys-Met-Ala, which is located 76 amino acids from the carboxyl terminus of chicken vimentin (Geisler and Weber, 1981), was synthesized by the solid phase triester procedure mainly as described by Miyoshi et al. (1980). The oligonucleotides were purified by gel filtration, followed by gel electrophoresis in 20%

or 25% polyacrylamide-urea gels. The probes were labeled with [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase. The sequences of the oligonucleotides were confirmed by DNA sequencing using a modification of the Maxam-Gilbert technique (1980), in combination with 5′ end nucleotide determination by thin layer chromatography of S1 nuclease-treated ^{32}P-labeled oligonucleotides.

Screening of the Chicken Genomic Library

The chicken Charon 4A genomic DNA library used was prepared by Dodgson et al. (1979) by partial digestion with Hae III and Alu I restriction enzymes, as described by Maniatis et al. (1978).

A. Screening with Synthetic Oligonucleotide Probes

Duplicate nitrocellulose filters representing 9×10^5 pfu's were prepared as described by Benton and Davis (1977) and prehybridized at 65°C for 3 hr in a solution containing 6X SET buffer (1X SET is 0.15 M NaCl, 30 mM Tris, pH 7.5, 1 mM EDTA), 5X Denhardt's solution [1X Denhardt's solution is 0.02% BSA, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone (Denhardt, 1966)] 0.5% NP-40 and 100 μg/ml sheared single stranded salmon testis DNA. Hybridization was carried out at 25°C for 48 hr in 6X SET buffer, 2X Denhardt's solution, 250 μg/ml tRNA, containing 4×10^6 cpm ^{32}P-labeled oligonucleotide probe per ml. Filters were washed four times at room temperature in 6X SSC for 20 min each and then twice at 25°C in 6X SSC for 30 min each. Filters were air-dried and exposed to Kodak XAR film with Dupont Lightening Plus intensifying screens.

B. Screening with the Vimentin cDNA

4×10^5 pfu's of the genomic DNA library were screened using nick-translated (Rigby et al., 1977) insert of the p5C5 vimentin cDNA clone. Filters were prepared and hybridized to ^{32}P-labeled insert according to Benton and Davis (1977).

DNA Isolation

Phage DNA was isolated essentially as described by Yamamoto et al. (1970). Plasmid DNA was isolated either as described by Clewell and Helinski (1972) or by Holmes and Quigley (1981). High molecular weight DNA was prepared from liver nuclei by a modification of the method described by Firtel and Bonner (1972). Restriction enzyme-generated DNA fragments were isolated from low melting temperature agarose gels mainly as described by Weislander (1979), or from normal agarose gels according to Brown et al. (1976). Oligonucleotides were isolated from 20% or 25% polyacrylamide-urea gels by the method described by Maxam and Gilbert (1977).

Subcloning of Genomic Vimentin Sequences into pBR322

Plasmids containing genomic vimentin sequences were constructed according to Mertz and Davis (1972) and Cohen et al. (1973) by ligation of EcoRI-BamHI-, BamHI-, and Hind III-digested fragments of the genomic λV8 clone with pBR322 which had been digested with the appropriate enzymes to generate complementary ends. The recombinant plasmids were then used to transform *E. coli* HB101 (Cohen et al., 1972). Nick-translated vimentin-specific sequences were used to select clones containing the desired fragments (Hanahan and Meselson, 1980).

Restriction Endonuclease Mapping

Restriction endonuclease cleavage sites were determined either by single and double digests with various restriction endonucleases or by partial digestion of end-labeled fragments according to Smith and Birnstiel (1976). Digestion products were resolved in conventional horizontal agarose gels.

RNA Blotting and Hybridization

RNA electrophoresis under denaturing conditions in the presence of formaldehyde was carried out according to Lehrach et al. (1977) as modified by Capetanaki et al. (1982). Gels were soaked in 20X SSC for 30 min and transferred to nitrocellulose with 10X SSC for 10–15 hr, using the transfer system described by Southern (1975).

Hybridizations of $\sim 5 \times 10^5$ cpm/ml ^{32}P-labeled nick-translated probes (Rigby et al., 1977) to filter-bound RNA were performed as described by Wahl et al. (1979) at 42°C for 15 hr. Following hybridizations, filters first were washed three times at room temperature with 2X SCC, 0.2% SDS, for 15 min each and then four times at 50°C with 0.2 X SCC, 0.2% SDS for 30 min each. Filters were dried and exposed to Kodak XAR film with intensifying screens. RNA hybridizations using the synthetic oligonucleotide probes were performed as described for the genomic library screening.

Acknowledgments

We are grateful to Dr. Richard Ogden for his help in the preparation of the synthetic oligonucleotide probes, Dr. Jerry B. Dodgson for the gift of the chicken genomic library and Drs. Eric Davidson and Norman Davidson for their helpful comments on the manuscript. We also thank Adriana Cortenbach and Ilga Lielausis for their technical assistance. This work was supported by grants from the National Institutes of Health, the National Science Foundation, the Muscular Dystrophy Association of America and by a Grant-in-Aid from the American Heart Association Greater Los Angeles Affiliate. Y. G. C. was also supported by a Muscular Dystrophy Association Postdoctoral Fellowship and J. N. by a National Science Foundation Predoctoral Fellowship. C. N. F. is a Lievre fellow of the American Cancer Society, California Division. E. L. is a recipient of a Research Career Development Award from the National Institutes of Health.

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Received August 10, 1983; revised September 14, 1983

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CHAPTER 3:

Differentiation of Murine Erythroleukemia Cells Results in the Rapid Repression of Vimentin Gene Expression

(Appeared in *The Journal of Cell Biology*, Vol. 99, pp. 306-314, 1984)

Differentiation of Murine Erythroleukemia Cells Results in the Rapid Repression of Vimentin Gene Expression

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ABSTRACT We show that vimentin filaments are present in undifferentiated Friend murine erythroleukemia cells, but are lost progressively to undetectable levels by 96 h of dimethyl sulfoxide-mediated differentiation. The amount of newly synthesized cytoskeletal vimentin is decreased dramatically by 24 h of induction, and is paralleled by a rapid loss of vimentin mRNA (~25-fold reduction at 96 h). Hence, disappearance of vimentin filaments in these cells appears to be regulated at the level of vimentin mRNA abundance. On the other hand, the levels of actin synthesis and actin mRNA remain essentially unchanged. The kinetics of vimentin mRNA reduction during dimethyl sulfoxide-mediated differentiation, and the levels of vimentin mRNA observed in the presence of hexamethylene-bisacetamide or hemin as inducers suggest that the cessation of vimentin expression precedes, but may be associated with commitment to terminal differentiation. Our results demonstrate the dynamic regulation of vimentin expression in mammalian erythropoiesis.

Elucidation of the events occurring in mammalian erythropoiesis has been facilitated by the study of murine erythroleukemia cell differentiation in vitro. Friend murine erythroleukemia (MEL)[1] cells originally were derived from Friend leukemia virus-infected tumor implants and proliferate rapidly in culture (13). Exposure to a variety of chemical agents, such as dimethyl sulfoxide (DMSO), hexamethylene-bisacetamide (HMBA), or butyric acid induces MEL cells to differentiate (14, 29, 48; for a review see reference 35). Among the characteristic phenomena observed during terminal differentiation of MEL cells are a maturation from a basophilic erythroblastic appearance to an orthochromatophilic normoblastic phenotype (14), a loss of proliferative capacity (12, 21), alterations in purine metabolism (46), increases in iron uptake and heme synthesis (14), an elevation of heme synthetic enzyme activities (10, 54), the induction of globin mRNAs (41,43,50), and the accumulation of hemoglobin (14). The tremendous induction of hemoglobin synthesis and accumulation in chemically-induced MEL cells, a distinctive feature of erythroid terminal differentiation in vivo, is effected by transcriptional activation of globin genes (2, 34, 44); the relative accumulation of globin mRNAs also may be modulated by the stabilization of these messages and/or by the destabilization of nonglobin mRNAs (2, 32, 56, 60).

[1] *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; HMBA, hexamethylene-bisacetamide; MEL, Friend murine erythroleukemia; TX-100, Triton X = 100.

Studies from this laboratory have demonstrated that vimentin is the major intermediate filament subunit in chicken erythrocytes (19). We have shown further that during chicken embryonic development, the expression of vimentin in circulating erythroid cells is regulated at the level of transcription and/or mRNA stabilization (5). An approximately 50-fold increase in the accumulation of vimentin mRNA is observed between 4-d primitive series cells and 15-d definitive series cells, and this increase appears to underlie similar changes at the protein level (5). In contrast, Dellagi et al. (7) have shown by immunofluorescence microscopy that vimentin is lost during human erythropoiesis in vivo. However, the loss of vimentin could not be correlated with a particular stage of erythroid development, as the presence of vimentin was variable in both early and late erythroblastic cells.

To define the mechanisms that regulate vimentin expression during mammalian erythropoiesis, we have examined the pattern of expression of this intermediate filament subunit in differentiating MEL cells. We have found that vimentin filaments disappear from the cytoplasm of DMSO-treated MEL cells, and that this disappearance results from a rapid decrease in vimentin synthesis. Furthermore, we demonstrate that the observed change in vimentin synthesis during induction is caused by a rapid and dramatic decrease in the amounts of steady state vimentin mRNA, indicating that vimentin filament expression in differentiating MEL cells is regulated primarily at the mRNA level. Our data suggest that the cessation of vimentin filament expression is an essential com-

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ponent of the terminal differentiation program in mammalian erythropoiesis.

MATERIALS AND METHODS

Cell Culture: Friend murine erythroleukemia cells were obtained from Dr. Barbara J. Wold (California Institute of Technology, Pasadena, CA) and were maintained in Dulbecco's modified Eagle medium (DME) supplemented with 15% fetal calf serum and penicillin/streptomycin at 37°C in an atmosphere of 95% air/5% CO_2. Cells were passaged every third day at dilutions of 1:100. For induction experiments, cultures were initiated at densities ranging from 5 × 10^4/ml to 2 × 10^5/ml, and incubated in the presence of 1.8% DMSO, 4 mM HMBA, or 75 μM bovine hemin (from a 10-mM stock solution, prepared as described by Ross and Sautner [51]) for the times indicated in each of the experiments. Inducers were added to culture media prior to the addition of cells. Cells from control cultures were harvested after 2–3 d. In general, cells were harvested while they were still in logarithmic growth (although cells induced in DMSO or HMBA for 96 h had just begun to reach plateau densities, as expected at this stage of MEL cell terminal differentiation [12, 21]). Growth kinetics were derived by estimation using a hemacytometer, and cell viability was confirmed by trypan blue exclusion.

Immunofluorescence Microscopy: Vimentin-containing intermediate filaments were visualized using a rabbit anti-chicken vimentin antiserum (17). Although this antiserum displays weaker reactivity with mammalian vimentin than with chicken vimentin, immunoprecipitation of [^{35}S]labeled MEL cell proteins (method B of reference 4) indicates that vimentin is specifically recognized in this system (data not shown).

MEL cells from different periods of induction were washed twice with Tris-buffered saline (140 mM NaCl, 5 mM KCl, 10 mM Tris-Cl, pH 7.5), resuspended in Tris-buffered saline, and allowed to settle onto Alcian Blue-treated coverslips at 4°C (19). Adherent cells were fixed for 2 min at room temperature in 2% formaldehyde, 140 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 20 mM PIPES, pH 7.0, and rinsed and permeabilized in Tris-buffered saline plus 0.5% Triton X-100 (TX-100) and 1 mM EDTA. Cells were reacted first with the rabbit anti-chicken vimentin antiserum at 1:40 dilution, and then with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:150 (Miles-Yeda Ltd., Rehovot, Israel). Coverslips were mounted in Tris-buffered saline and viewed in a Leitz phase/epifluorescence microscope using a 63 × objective.

Metabolic Labeling with [^{35}S]Methionine and Two-dimensional Gel Electrophoresis: For each time point examined, 2 × 10^6 cells were pelleted by centrifugation and washed in 5 ml Dulbecco's modified Eagle medium depleted of methionine and supplemented with 15% dialyzed fetal calf serum, with or without 1.8% DMSO. Cells were resuspended in 5 ml of this medium plus ~100 μCi [^{35}S]methionine (Amersham Corp., Arlington Heights, IL, ca. 1,100–1,200 Ci/mmol) and incubated at 37°C for 1 h. After labeling, cells were placed on ice, washed with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution (EBSS), and resuspended in 100 μl 100 mM KCl, 300 mM sucrose, 20 mM PIPES, 5 mM Mg acetate$_2$, 5 mM EGTA, 0.5% TX-100, pH 6.8 (cytoskeleton buffer; 39). Lysis was allowed to proceed for 5 min at 0°C, and insoluble "cytoskeletal" fractions were pelleted by centrifugation. After removal of detergent-soluble phases, pellets were resuspended in cytoskeleton buffer plus 2.5% 2-mercaptoethanol, saturated with urea. The TX-100-insoluble residues are defined operationally as cytoskeletal fractions. TX-100 fractionation is necessary for two-dimensional gel analysis of mammalian vimentin, as in our gel system the large quantities of tubulin (mostly soluble under these lysis conditions) obscures the identification of vimentin. TX-100 insoluble ^{35}S-labeled vimentin therefore represents newly synthesized and newly assembled cytoskeletal vimentin (3, 39).

Protein-incorporated ^{35}S-radioactivity was determined from total lysates as well as from TX-100 soluble and insoluble fractions. Briefly, aliquots were incubated in 1 N NaOH, 4 mg/ml methionine, and 0.4 mg/ml BSA at 37°C for 10 min to hydrolyze methionyl-tRNA complexes, precipitated with trichloroacetic acid, and collected on Whatman GF/C filters (Whatman Laboratory Products, Inc., Clifton, NJ). Radioactivity was determined by liquid scintillation counting in Aquasol-2 (New England Nuclear, Boston, MA). Typically 12–14% of protein-incorporated ^{35}S-radioactivity was found in the cytoskeletal fraction. For electrophoretic analysis, the amounts of sample used were normalized to total cell lysate protein ^{35}S-radioactivity.

Two-dimensional gel electrophoresis was performed as described by O'Farrell (42) and modified by Hubbard and Lazarides (23). Resolving gels in the second dimension contained 12.5% acrylamide and 0.1% bisacrylamide. After electrophoresis, gels were fixed, impregnated with Enhance (New England Nuclear), and subjected to fluorography using preflashed Kodak XAR-5 film (26). ^{35}S-labeled vimentin was identified on the basis of its co-migration with purified bovine lens vimentin (40).

RNA Isolation: Cells were harvested by centrifugation at appropriate times, washed twice with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution, quickly frozen in liquid N_2, and stored at −80°C. Total cellular RNA was isolated by the method of Chirgwin et al. (6). Frozen cell pellets (each containing up to ~4–5 × 10^8 cells) were thawed into 27 ml 5 M guanidinium thiocyanate, 50 mM Tris-Cl, pH 7.5, 50 mM EDTA, and 5% 2-mercaptoethanol. The resulting viscous DNA was sheared by at least 10 strokes in a Dounce homogenizer (with a tightly fitting pestle), and by 5 additional strokes after the addition of 3 ml 30% sodium lauryl sarcosine. Homogenates were layered over 8 ml cushions of 5.7 M CsCl, 1 mM EDTA, pH 8.0, and RNA was pelleted by centrifugation for 20–24 h at 39,000 rpm in a Beckman 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 20°C. Pellets were resuspended in water, precipitated twice with ethanol, washed once with 70% ethanol, and dried. The recovery of RNA by this method is quantitative if care is taken to shear sufficiently the cellular DNA (which traps RNA); we find that recovery of RNA from cells at a given stage of induction is constant irrespective of cell number (between ~1.5 × 10^8 and ~4.5 × 10^8 cells/30 ml homogenization buffer). Poly(A)$^+$-enriched RNA was isolated by two rounds of oligo(dT)-cellulose chromatography (1). Within an induction experiment, the conditions of oligo(dT)-cellulose chromatography for each sample were maintained as identical as possible. In this manner, a constant fraction of total RNA was recovered as poly(A)$^+$ RNA (generally 2% recovery), regardless of the stage of MEL cell differentiation.

RNA Electrophoresis, Blotting to Nitrocellulose, and Hybridization: RNA was separated on 1.1% agarose gels under denaturing conditions in the presence of formaldehyde (30), essentially as described by Goldberg (16). Following electrophoresis, gels were soaked in 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM Na-citrate) and transferred to nitrocellulose in 10 × SSC according to the method of Southern (58). Filters were washed briefly in 2 × SSC, air dried, and baked in vacuo at 80°C for 2–3 h.

^{32}P-labeled DNA probes were utilized to detect specific filter-bound RNA sequences. For the detection of vimentin RNA, the ~0.5 kilobase (kb) insert from p5C5, a chicken vimentin cDNA plasmid (5) was isolated (62) and nick-translated (49) using α-^{32}P-labeled deoxynucleoside triphosphates (ca. 800 Ci/mmol, New England Nuclear). Detection of β-globin RNA sequences was facilitated by nick-translation of pMB-βG2, a recombinant mouse β^{major}-globin genomic plasmid (25), whereas actin sequences were identified using a ^{32}P-labeled actin cDNA plasmid derived from chicken smooth muscle. This actin cDNA plasmid (containing a 1.9 kb-insert) was isolated by screening a chicken gizzard cDNA library with a sea urchin actin probe (55). Its identity as an actin cDNA was confirmed by positive hybrid-selected translation (data not shown).

Filter-bound RNA was hybridized to 5 × 10^5 cpm/ml nick-translated probe at 42–44°C for 20–24 h, as described by Wahl et al. (61) in the absence of dextran sulfate. Filters then were washed twice in 2 × SSC, 0.2% SDS at room temperature for 20–30 min each, and four times in 0.1 × SSC, 0.2% SDS at 42–44°C for 30 min each, and air dried. Blots were exposed to preflashed Kodak XAR-5 film with DuPont Lightning-Plus intensifying screens.

Quantitation of RNA Blot Hybridizations: The relative levels of specific RNA species during MEL cell differentiation were quantitated by determining densities of autoradiographic signals from RNA blot hybridizations. For this technique to be reliable and effective, however, a number of variables were controlled. First, for every experiment requiring quantitation, the RNA sample containing the highest amount of the sequence of interest was diluted and included in the same gel containing the other experimental samples. For example, vimentin mRNA quantitation was standardized by using control RNA diluted to 1.0-, 0.5-, 0.25-, 0.1-, and 0.05-equivalents. For actin mRNA, control RNA was diluted to 1.0-, 0.8-, 0.6-, and 0.4-equivalents, whereas for globin mRNA quantitation, RNA from cells induced for 96 h in DMSO was diluted to 1.0-, 0.5-, 0.25-, 0.1-, and 0.05-equivalents. Hence, concentration standards were treated identically as the experimental samples were through electrophoresis, transfer, hybridization, washes, exposure, and photographic development.

Second, deviations from linearity of film response to signal were taken into account. The inclusion of standards with each blot to be quantitated controlled for reciprocity failure at low intensity levels. However, hybridization bands that expose the film to or beyond saturation cannot be quantitated. The effects of nonlinear film response were minimized by preflashing film to an optical density of 0.15 at 540 nm (26), and by exposing films within linear range for several periods of duration. Autoradiograms were scanned with a densitometer, and peak areas were determined using a computer digitizer.

Fig. 1 shows an example of a standardization for vimentin mRNA. The autoradiogram of Fig. 1*A* was from the same blot (and same film) as the one of Fig. 4*A*. The graph in Fig. 1 *B* shows the relationship between peak area and relative level of vimentin mRNA. Although the overall response is not linear, this type of curve is typical.

RESULTS

Disappearance of Vimentin Filaments from DMSO-induced MEL Cells

Indirect immunofluorescence microscopy using an antivimentin antiserum revealed that vimentin filaments are lost from differentiating MEL cells. Cells were cultured in control medium or for 24, 36, 48, and 72 h in the presence of 1.8% DMSO and prepared for immunofluorescence microscopy. Representative micrographs are shown in Fig. 2. MEL cells grown in control medium exhibit cytoplasmic vimentin filaments. Upon incubation with DMSO, however, vimentin-specific fluorescence progressively diminishes. After 24 h in DMSO, vimentin filaments are still visible in most cells, but their presence is more difficult to detect than in control cells. Longer periods of DMSO induction result in a further diminution of detectable vimentin, and filaments generally are not visible after 48 h of incubation. Diffuse cytoplasmic fluorescence is also observed in all cells; this is due to nonspecific staining, as it is observed when preimmune serum is used as the primary antibody (data not shown), and is present irrespective of the cellular vimentin content (see below).

The loss of vimentin filaments in differentiating MEL cell cultures appears to be uniform; for a given time point, distinct subpopulations of vimentin-positive and vimentin-negative cells are not seen. The presence of apparently vimentin-negative cells among positive cells (Fig. 2) is due primarily to the positions of cells outside the plane of focus. However, visualization of cells exposed to DMSO for 48 or 72 h occasionally reveals a cell displaying vimentin filaments (see Fig. 2). These are rare cases (<1%), and most likely represent cells that did not respond to the inducer. By this qualitative assay, the disappearance of detectable vimentin filaments occurs in differentiating MEL cells between 24 and 48 h of DMSO-mediated induction, a time that corresponds to the onset of significant hemoglobin accumulation (31, 36; see also below).

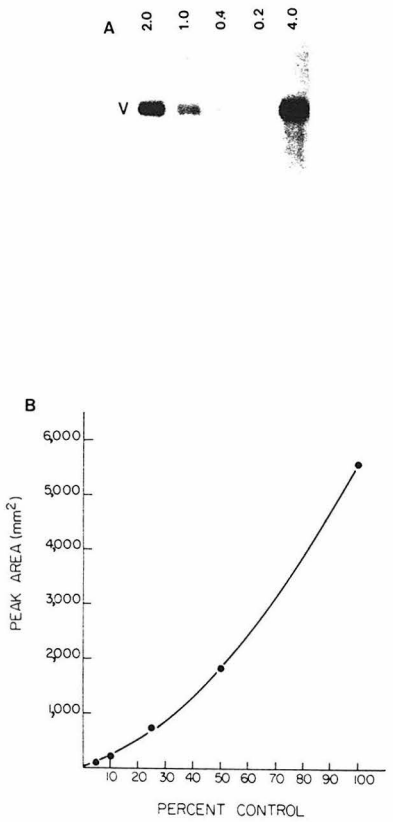

FIGURE 1 Standardization of vimentin RNA hybridization. (*A*) Autoradiogram of an RNA blot containing 4.0, 2.0, 1.0, 0.4, and 0.2 μg control MEL cell poly(A)$^+$ RNA, hybridized to the vimentin cDNA probe. (*B*) Quantitation of the blot in *A*. Vimentin mRNA peaks were scanned and integrated with a computer digitizer. The data are expressed as peak area vs. percent control, with 100% corresponding to 4 μg poly(A)$^+$ RNA.

Analysis of Newly Synthesized Cytoskeletal Vimentin in Differentiating MEL Cells

MEL cells differentiating in response to DMSO rapidly and extensively reduce the synthesis of vimentin, and consequently cease the de novo assembly of vimentin filaments. Although ^{35}S-labeled vimentin is present in cytoskeletons from control MEL cells (Fig. 3*A*), after 24 h of induction newly synthesized cytoskeletal vimentin is barely detectable (Fig. 3*B*), and at 36 and 72 h of culture in DMSO (Fig. 3, *C* and *D*), remains at or below detection limits. Since de novo assembly of vimentin filaments ceases rapidly, the disappearance of filaments in MEL cell differentiation may result from dilution by cell division (see Discussion).

Immunoprecipitation of newly synthesized cytoskeletal vimentin and subsequent one-dimensional gel analysis also yields a pattern similar to that seen in Fig. 3; we observe a slight decrease in ^{35}S-labeled vimentin after 12 h of induction, and at 24 h and all subsequent time points vimentin was nearly or completely absent (data not shown). Examination of ^{35}S-labeled vimentin from total cell lysates by immunoprecipitation revealed that vimentin synthesis is diminished at 24 h of incubation in DMSO and thereafter (data not shown). Hence, the reduction in newly synthesized cytoskeletal vimentin parallels the rapid and extensive repression of vimentin synthesis. These observations are consistent with previous studies from this laboratory, which demonstrated that vimentin assembly in chicken erythroid cells occurs rapidly and post-translationally from a saturable pool, but with assembly appearing not to be regulated at this level (3, 39).

The data in Fig. 3 indicate that the reduction in newly synthesized cytoskeletal vimentin (and consequently the reduction in newly synthesized total cellular vimentin) is specific, even though overall protein synthesis is reduced in differentiating MEL cells (57). Several other ^{35}S-labeled proteins are present in fairly constant amounts throughout the 72-h induction period, most notably actin. ^{35}S-labeled actin was also maintained at constant levels in total cell lysates (data not shown).

Rapid Reduction in Vimentin mRNA Levels During the Maturation of MEL Cells

The data presented thus far show that the disappearance of vimentin filaments in differentiating MEL cells is mediated by a decrease in vimentin synthesis. To analyze steady state vimentin mRNA levels during induction, we performed quantitative RNA blot analysis of poly(A)$^+$ RNA derived from cells undergoing DMSO-induced differentiation, using a chicken vimentin cDNA as probe. This cDNA probe, designated p5C5, corresponds to the 5′ region of the chicken vimentin gene (5). When p5C5 was hybridized to RNA blots

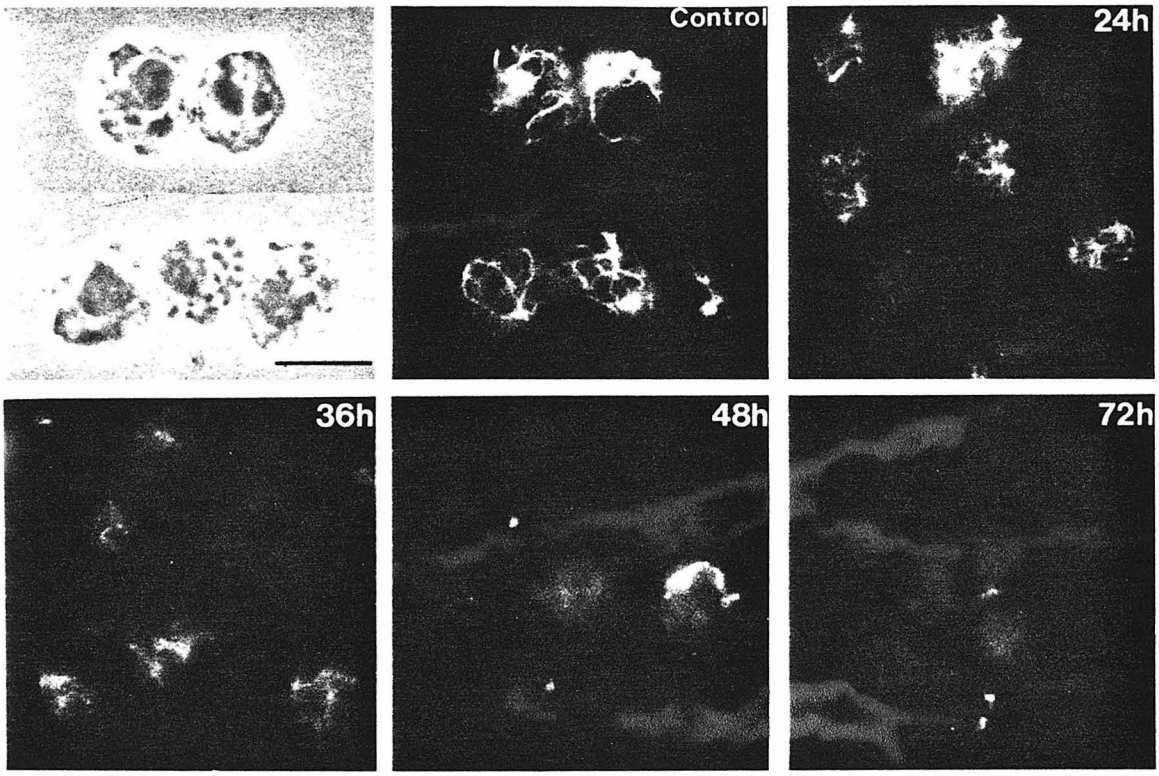

FIGURE 2 Disappearance of vimentin filaments during DMSO-induced MEL cell differentiation. Vimentin filaments in MEL cells were visualized by indirect immunofluorescence microscopy using an antivimentin antiserum. The left-most panel of the top row is a phase contrast image of the same field shown in the control fluorescence panel. Cells were examined at 24, 36, 48, and 72 h of induction in 1.8% DMSO, as indicated. Note the presence of a rare vimentin-positive cell at 48 h of differentiation (see text). Bar, 10 μm.

containing poly(A)$^+$ RNA from total 1-d-old neonatal mice, only one species with an approximate molecular length of 2.1 kb was detected (data not shown), in agreement with the data of others for the mature mammalian vimentin mRNA (8, 45).

MEL cells were cultured in the presence of 1.8% DMSO and were harvested after 12, 24, 36, 48, 72, and 96 h of induction; untreated cells were maintained in control medium. Poly(A)$^+$ RNA was isolated from each sample and analyzed by RNA blot hybridization using p5C5 as probe. An autoradiogram of a representative RNA blot is shown in Fig. 4*A*. A 2.1-kb RNA band corresponding to vimentin mRNA is clearly detected in poly(A)$^+$ RNA from control MEL cells. After 12 h of culture in DMSO, this band is somewhat reduced, and declines rapidly between 12 and 96 h of induction. Fig. 4*B*, which represents data derived from two independent induction experiments, shows the quantitation of relative vimentin mRNA levels through 96 h of DMSO induction. After 12 h of exposure to inducer, vimentin mRNA is ~70% of the control level, and falls rapidly thereafter to approximately 30% at 24 h, 10% at 48 h, and 4% at 96 h of differentiation. The results demonstrate that vimentin expression in differentiating MEL cells is regulated by the steady state level of vimentin mRNA.

The data of Fig. 4 show relative vimentin mRNA levels normalized to total cellular RNA, since the fraction of poly(A)$^+$ RNA that we recover from total RNA is constant throughout the induction period (see Materials and Methods). However, the amount of total RNA recovered per cell decreases as differentiation of MEL cells progresses. From control cultures, we obtain ~1.5–1.8 mg RNA/10^8 cells, and this value declines steadily to ~0.4–0.5 mg RNA/10^8 cells after 96 h of culture with DMSO, consistent with the results of others (33, 57). Hence, the kinetics of vimentin mRNA reduction per cell is actually more rapid than shown in Fig. 4*B*, and the overall extent of this loss is greater (~1% control levels per cell at 96 h of induction). The ~100-fold reduction in vimentin mRNA levels per cell therefore results from both a specific decrease in vimentin mRNA and a general decline in total cellular RNA.

Quantitation of Actin and β-Globin mRNA Levels in Differentiating MEL Cells

To characterize further the specific reduction in vimentin mRNA during DMSO-induced differentiation, we performed as controls quantitative RNA blots using probes for actin and β-globin. As in Fig. 4*A*, equivalent amounts of poly(A)$^+$ RNA from different time points of DMSO induction were electrophoresed and transferred to nitrocellulose. Fig. 5*A* is an autoradiogram of a blot hybridized to a ^{32}P-labeled chicken actin cDNA, and the quantitation of this blot is shown in Fig. 5*B*. From Fig. 5, *A* and *B*, it is clear that the abundances of actin mRNA in poly(A)$^+$ RNA from differentiating MEL cells remain essentially constant through 96 h of culture in the presence of DMSO. The results show that the constant level of actin expression in MEL cells is regulated at the mRNA level.

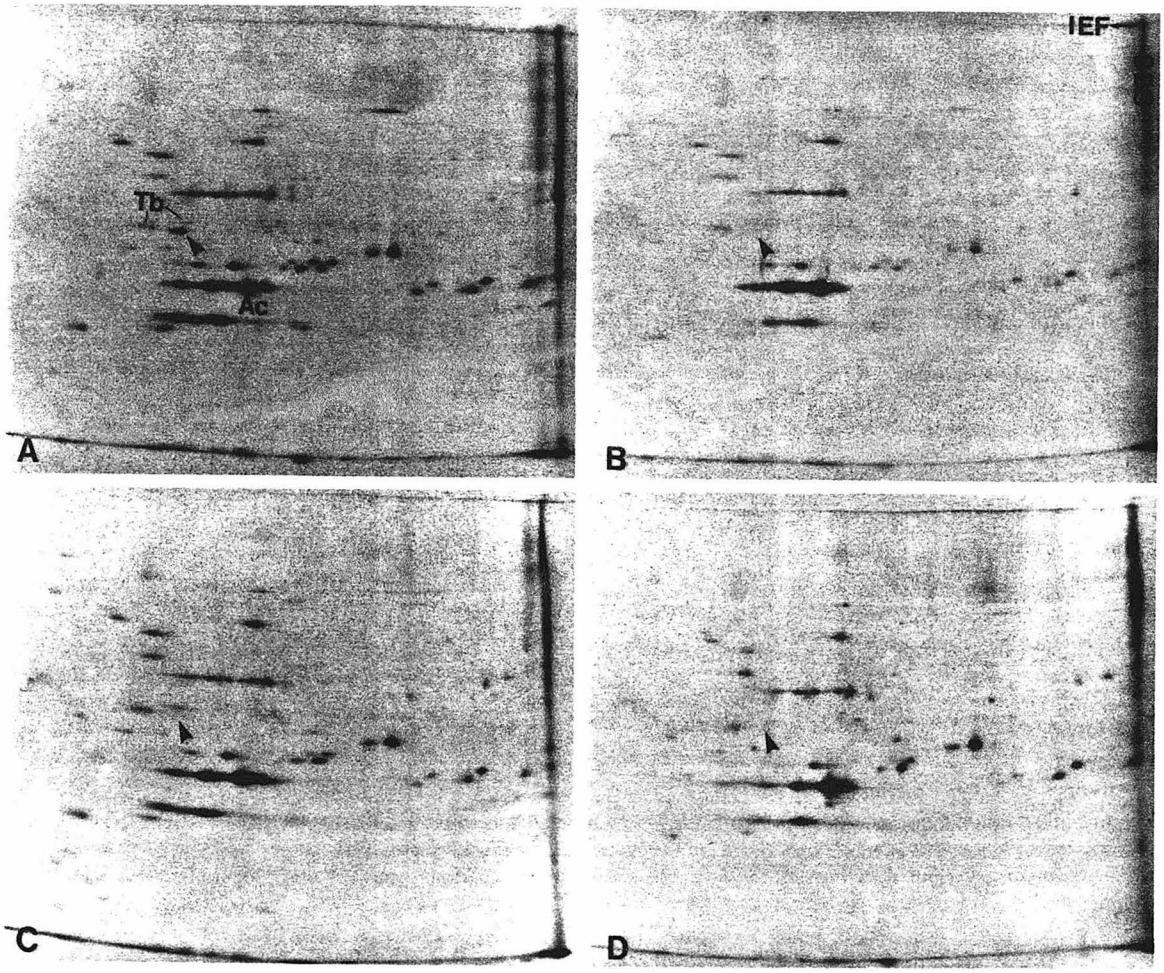

FIGURE 3 Two dimensional gel analysis of newly synthesized cytoskeletal vimentin during MEL cell differentiation. Control cells and cells cultured in the presence of 1.8% DMSO for 24, 36, or 72 h were pulse-labeled for 1 h with [^{35}S]methionine. Cytoskeletal fractions were prepared and analyzed by two-dimensional gel electrophoresis and fluorography (see Materials and Methods). (*A*) control; (*B*) 24 h; (*C*) 36 h; and (*D*) 72 h. Arrowheads demarcate vimentin, as determined by its co-migration with a purified bovine vimentin standard. *Tb*, tubulins; *Ac*, actin.

The kinetics of globin mRNA induction under our culture conditions was assessed by hybridizing RNA blots with a ^{32}P-labeled recombinant plasmid containing mouse genomic β^{major}-globin sequences (25). The results of such experiments are presented in Fig. 5, *C* and *D*. After a ~24-h lag period, a significant increase in β-globin mRNA is observed between 24 and 36 h of culture in the presence of DMSO, and there is an overall ~20-fold increase of β-globin mRNA over the basal level at 96 h of induction. The pattern of β-globin mRNA induction observed in this study is similar to the patterns observed by other investigators (32–34, 41, 51). The amount of β-globin mRNA in control MEL cells is reproducibly higher than in cells incubated for 12 or 24 h in DMSO, and most likely is due to the accumulation (after 2–3 d of culture) of a low percentage of spontaneously differentiating cells in our control cultures (14; see also reference 35). At 48 h of DMSO-stimulated differentiation, β-globin mRNA increases ~5-fold over basal levels (see Fig. 5*D*), whereas vimentin mRNA already has fallen to approximately one-tenth of control amounts (Fig. 4*B*). The decrease in vimentin mRNA levels therefore precedes the major accumulation of β-globin mRNA.

Effects of HMBA and Hemin on Vimentin mRNA Levels in MEL Cells

A variety of chemical agents other than DMSO can induce MEL cells to differentiate. For example, HMBA is a particularly potent inducer, and causes terminal differentiation of MEL cells, as does DMSO (48). On the other hand, incubation of MEL cells with hemin causes the rapid accumulation of globin mRNA, but does not induce other changes characteristic of terminal differentiation (22, 33, 51). We therefore tested the effects of these two agents on the levels of vimentin mRNA in MEL cells.

After 96 h of exposure to 4 mM HMBA, MEL cells exhibit an extensive reduction in vimentin mRNA, as shown by RNA blot analysis (Fig. 6*A*). The levels at 96 h of HMBA-mediated induction are similar to those seen with 1.8% DMSO after 72 h. Fig. 6*C* is a control, showing the large induction at 96 h of β-globin mRNA in response to HMBA. Hence, MEL cells dramatically decrease the steady state level of vimentin mRNA during both HMBA- and DMSO-mediated differentiation.

Incubation of MEL cells with 75 μM hemin for 72 h causes

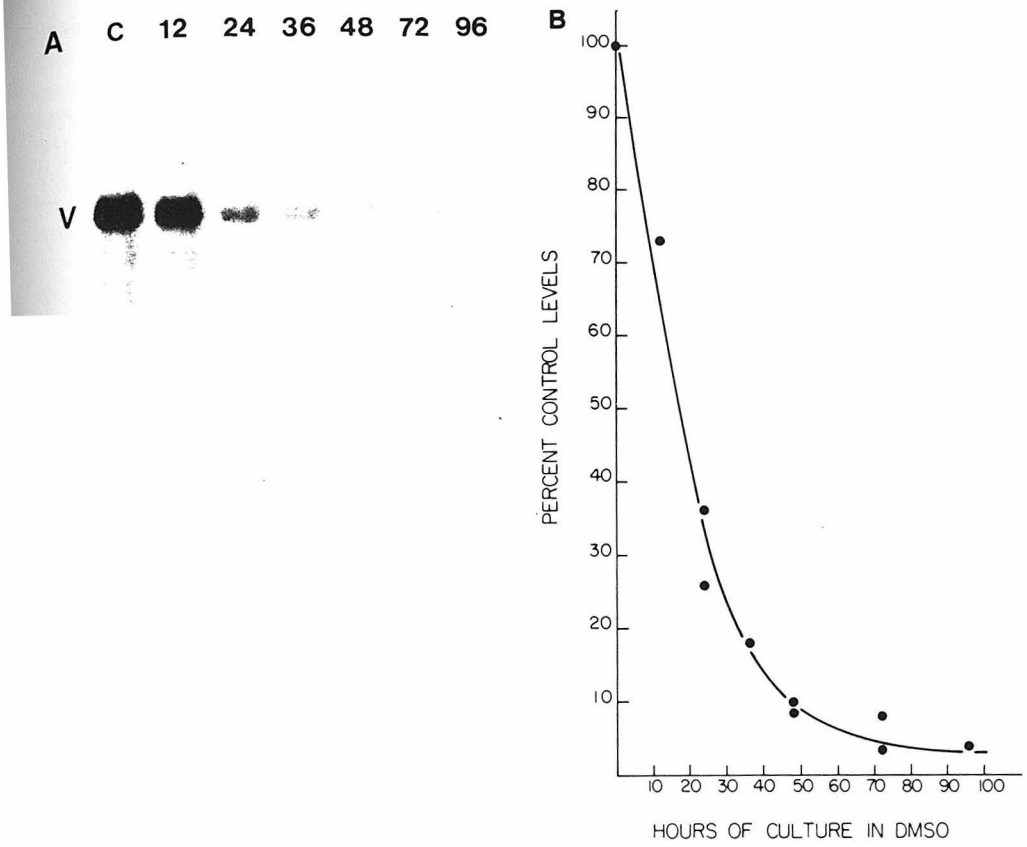

FIGURE 4 Analysis of vimentin mRNA levels during MEL cell differentiation. 4 μg of poly(A)$^+$ RNA from control cells or cells cultured in 1.8% DMSO for 12, 24, 36, 48, 72, and 96 h were electrophoresed in the presence of formaldehyde, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled chicken vimentin cDNA probe. (*A*) Autoradiogram of a representative blot. C denotes poly(A)$^+$ RNA from control cells; numbers indicate hours of culture in the presence of 1.8% DMSO from which poly(A)$^+$ RNA was derived. *V* marks the position of the 2.1-kb mouse vimentin mRNA. (*B*) Quantitation of vimentin mRNA levels during induction. The data in this graph were derived from two independent experiments from blots using either 4.0 or 2.2 μg RNA per lane, and vimentin mRNA levels were quantitated as described in Materials and Methods. The values correspond to relative quantities of vimentin mRNA normalized to cellular poly(A)$^+$ RNA content (see text), as compared with the control cell vimentin mRNA level.

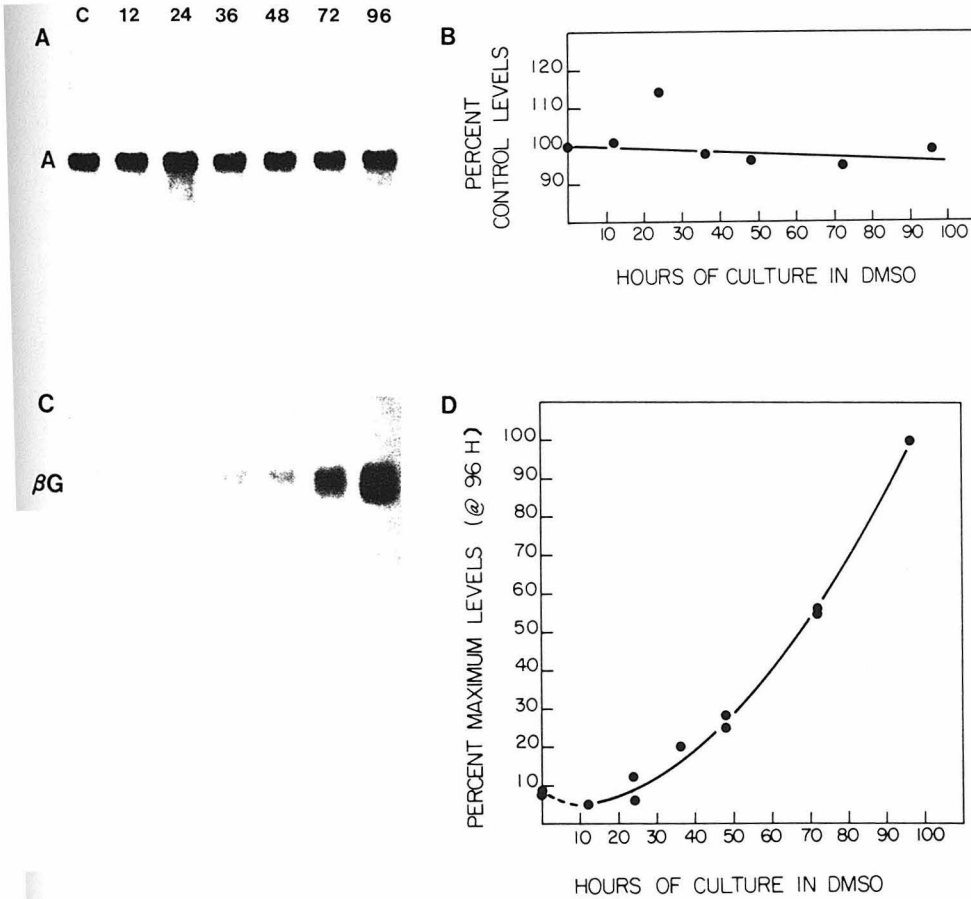

FIGURE 5 Actin and β-globin mRNA in differentiating MEL cells. Poly(A)$^+$ RNA from MEL cells was prepared for quantitative RNA blot hybridization as described in Materials and Methods. (*A*) Autoradiogram of a blot containing 3 μg of each poly(A)$^+$ RNA, which was hybridized to a chicken actin cDNA probe. (*B*) Quantitation of actin mRNA levels from the RNA blot shown in *A*. (*C*) Autoradiogram of a blot (4 μg poly(A)$^+$ RNA per lane) hybridized to a mouse β^{major}-globin probe. (*D*) Quantitation of β-globin mRNA levels during MEL cell differentiation, expressed as percentages of the level observed at 96 h of DMSO-mediated differentiation. The data in this graph were derived from two independent experiments. *C*, control cell poly(A)$^+$ RNA; *12, 24, 36, 48, 72, 96*, hours of culture in 1.8% DMSO. *A*, actin mRNA; *βG*, β-globin mRNA.

a less extensive change in vimentin mRNA, as compared with the effects of HMBA or DMSO (Fig. 6 *B*). Quantitation of an RNA blot similar to the one shown in Fig. 6 *B* indicates that vimentin mRNA is reduced to ~50% of control amounts. Fig. 6 *D* demonstrates a ~4–5-fold increase in β-globin mRNA in response to hemin, as reported by others (33, 51). By

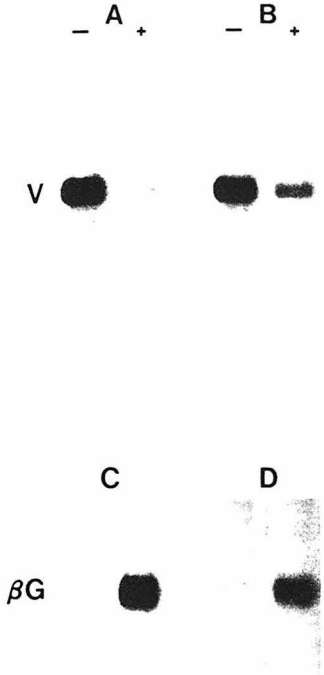

FIGURE 6 Effects of HMBA and hemin on MEL cell vimentin and β-globin mRNA levels. (*A*) Autoradiogram of an RNA blot containing poly(A)$^+$ RNA (4 μg each lane) from cells cultured in the absence (−) or presence (+) of 4 mM HMBA for 96 h, hybridized to the vimentin cDNA probe. (*B*) RNA blot analysis of vimentin mRNA from cells cultured in the absence (−) or presence (+) of hemin for 72 h (4 μg per lane). The hybridization seen with RNA from hemin-treated cells represents ~50% of control vimentin mRNA levels (see text). (*C*) RNA blot as in *A*, hybridized to the β-globin-specific probe. (*D*) Detection of β-globin mRNA in a blot similar to the one in *B*, except that 2.2 μg poly(A)$^+$ RNA was used per lane, V, vimentin mRNA; βG, β-globin mRNA.

immunofluorescence microscopy, nearly all cells cultured in the presence of 75 μM hemin for 3 d possess vimentin filaments (data not shown), indicating that most, if not all cells are responding similarly to this inducer with regard to vimentin expression. The response observed with hemin suggests that the more extensive reduction in vimentin mRNA observed in DMSO- and HMBA-treated cells is an event related to terminal differentiation (see Discussion).

DISCUSSION

Disappearance of Vimentin Filaments in Differentiating MEL Cells is Regulated at the mRNA Level

In this study we demonstrate that vimentin filaments disappear from differentiating MEL cells, and this disappearance is preceded by a rapid cessation of vimentin synthesis and assembly into the cytoskeleton (see below). Moreover, the level of vimentin synthesis is regulated primarily by vimentin mRNA levels, as the pattern of newly synthesized cytoskeletal vimentin during differentiation is paralleled by a similarly dramatic decrease in vimentin mRNA. The expression of a number of other proteins, most notably actin, is constant during chemically-induced MEL cell differentiation, indicating that the rapid and extensive repression of vimentin mRNA levels is a result of the specific regulation of vimentin gene expression.

Vimentin mRNA is reduced ~3-fold after 24 h of culture in 1.8% DMSO, and ~25-fold after 96 h (Fig. 4). Normalization of these values to cell number reveals that vimentin mRNA decreases to ~1% control levels per cell at 96 h of induction. This reduction results in a rapid decrease in newly synthesized cytoskeletal vimentin (Fig. 3). However, by immunofluorescence microscopy vimentin filaments are still detectable (with weaker fluorescence intensity) through 36 h of differentiation (Fig. 2). Vimentin filaments have been shown to be stable cellular constituents in other systems (3, 38). For example, in Ehrlich ascites tumor cells, the half-life of vimentin is similar in magnitude to the population doubling time (38); the decay of vimentin therefore is very slow compared with the mitotic rate of these cells. As the doubling time of MEL cells under our conditions of DMSO induction is 14–16 h, our data suggest that the loss of filaments is facilitated by a rapid turn-off of vimentin synthesis with continual dilution of existing filaments by cell division. It is also possible that the reduction in vimentin filaments during differentiation is accelerated by an increased rate of vimentin turnover.

Regulation of Vimentin Gene Expression

Our data demonstrate a dramatic repression of vimentin mRNA levels during MEL cell maturation. However, we do not know as yet the relative contributions toward this phenomenon by changes in vimentin gene transcription and post-transcriptional processing and turnover. Reduced transcription may result from both specific repression of the vimentin gene and an overall reduction of RNA synthesis, as the synthesis and accumulation of RNA in DMSO-treated MEL cells decreases during differentiation (57). In vitro nuclear transcription studies (20, 37) and/or pulse-labeling in vivo, with subsequent detection of newly synthesized vimentin transcripts are needed to demonstrate directly transcriptional regulation of the vimentin gene in this system. These studies also may detect any significant changes in transport of mature vimentin mRNA from the nucleus to the cytoplasm. The turnover rate of vimentin mRNA during inducer-mediated differentiation of MEL cells also may be increased, thereby accelerating the removal of vimentin mRNA from differentiating cells. Pulse-chase studies should elucidate the kinetics of vimentin mRNA turnover during MEL cell differentiation.

We have shown previously a tremendous induction of vimentin mRNA accumulation during chicken embryonic erythropoiesis (5). In chicken erythroid cells, two vimentin mRNA species of 2.0 and 2.3 kb are expressed at low levels in 4-d primitive series cells, and a specific 40–50-fold higher level of the 2.0-kb mRNA is observed by 15 d of development in definitive series cells. The increase in steady state vimentin mRNA during chicken embryonic erythropoiesis underlies similar changes observed at the protein level (5). Hence, although vimentin expression in chicken erythropoiesis in vivo and mammalian erythropoiesis in vitro are both regulated transcriptionally and/or post-transcriptionally, in the nucleated erythroid cells of chickens the regulation is positive, whereas in differentiating MEL cells the regulation is negative. In both cases, however, the change in magnitude of steady state vimentin mRNA is large (~50- to 100-fold). Such a comparison of vimentin mRNA levels during avian and mammalian erythropoiesis illustrates that vimentin gene expression can be dynamically regulated in both positive and negative fashions. The contrast between these two systems further suggests that the regulation of vimentin expression is an essential feature of each of the respective terminal differentiation programs, and the differences observed indeed may reflect the functional requirements of these cells (see below).

Relationship of Vimentin mRNA Repression to Commitment of MEL Cells to Terminal Differentiation

After a defined period in inducer, MEL cells acquire the capacity to continue in their maturation program in the

absence of chemical inducer; this characteristic represents commitment to terminal differentiation (12, 21). In the presence of DMSO, the major onset of MEL cell commitment appears to occur after 20–24 h of induction, at which time ~30% of the cells are committed; the appearance of committed cells increases rapidly thereafter, and at 48 h of induction reaches a plateau level of ~90% (21). Qualitatively similar results for DMSO-induced differentiation and commitment have been reported by others (41). By comparison, vimentin mRNA levels are reduced rapidly very early in DMSO-mediated induction; a significant decrease (to ~70% control levels) is observed at 12 h, the earliest time point examined (Fig. 4*B*). The kinetics of this repression indicates that the decline in vimentin mRNA precedes commitment of MEL cells to terminally differentiate, as vimentin mRNA levels are reduced ~3-fold by 24 h (Fig. 4*B*), corresponding to a time when a detectable fraction of committed cells is only just beginning to appear in the population (21, 41).

Two lines of evidence suggest that the decrease in vimentin mRNA expression in MEL cells is an event associated with terminal differentiation, even though this reduction temporally appears to precede commitment. First, HMBA, an agent that induces commitment and terminal differentiation (11), also causes a large repression of vimentin mRNA (Fig. 5*A*). Second, exposure to hemin for 72 h results in a comparatively smaller change in vimentin mRNA abundance (~50% of control, see Fig. 5*B*). MEL cells treated with hemin display a rapid (within ~6 h) increase in globin mRNA (22, 41, 51), but do not show changes characteristic of terminal differentiation (22), such as limited proliferative capacity (i.e., commitment; 12, 21), induction of cytidine deaminase activity (46), or induction of the chromatin-associated protein, IP_{25} (24). Our observations show that the precipitous decline in vimentin mRNA is an early event in MEL cell differentiation, and this event precedes, but may be associated with commitment to terminal differentiation. A conclusive answer to this subject awaits further experimentation with inhibitors of MEL cell differentiation (see reference 35) and the study of variant MEL cell lines resistant or hypersensitive to induction (for example, see reference 52).

Inducer-mediated MEL Cell Differentiation as a Model System for Vimentin Expression in Mammalian Erythropoiesis

Studies of human hematopoiesis in vivo have shown that vimentin expression is lost during erythropoiesis, although the stage at which this event occurs could not be ascertained (7). Furthermore, the loss of vimentin during the erythroblastic stages of erythropoiesis is maintained in the mature circulating erythrocyte, and no other intermediate filament subunits are detected (7). Intermediate filaments have never been described in the mature mammalian erythrocyte. Examination of the regulatory mechanisms involved in vimentin gene expression during MEL cell differentiation should facilitate the understanding of this phenomenon in vivo.

Since the function of intermediate filaments has not been determined directly (27, 28), the significance of the repression of vimentin expression in mammalian erythropoiesis is unclear. However, electron microscopic examination of nucleated chicken erythrocytes reveals a transcytoplasmic network of vimentin filaments that appears to anchor the centrally located nucleus (18). We speculate that the loss of vimentin filaments during mammalian erythropoiesis facilitates enucleation, as the presence of intermediate filaments at this late stage of development physically may hinder this process (for example, see reference 47). The repression of vimentin synthesis early in MEL cell differentiation may reflect the necessity in vivo of allowing a sufficient number of cell divisions to dilute existing filaments.

Generalization of MEL Cell Regulation of Vimentin Expression to Other Terminally Differentiating Cells

We have proposed previously that vimentin expression in terminally differentiating cells is regulated at the level of mRNA abundance (5). For example, neurons substitute vimentin completely with the neuron-specific intermediate filament protein during their differentiation (59). In other cell types, vimentin is replaced only partially by the cell-specific intermediate filament protein, such as desmin in muscle differentiation (15), and may be co-expressed with a cell type-specific intermediate filament subunit in the mature cell (e.g., chicken skeletal muscle: desmin [17], rat and chicken astrocytes: glial fibrillary acidic protein [59, 63], some mouse retinal neurons: neurofilament protein [9]). Our observation that the loss of vimentin filaments in differentiating MEL cells is regulated primarily by mRNA abundance suggests that the complete or partial repression of vimentin expression in other differentiating cell types is also controlled at the mRNA level.

We thank Dr. Barbara J. Wold for providing the murine erythroleukemia cells used in this study, Drs. Moses V. Chao and Richard Axel for their gift of pMB-βG2 plasmid DNA, Dr. W. James Nelson for supplying purified bovine lens vimentin standards, Dr. Bruce L. Granger for his advice regarding immunofluorescence microscopy, and Dr. Jean-Paul Revel for the use of his computer facilities. Drs. Wold, Nelson, Granger, and Dr. Randall T. Moon provided stimulating discussion throughout the course of this study. We are grateful to Drs. Granger and Moon for their comments on the manuscript. Ilga Lielausis and Adriana Cortenbach provided expert technical assistance. This work was supported by grants from the National Institutes of Health, the National Science Foundation, the Muscular Dystrophy Association, and a grant-in-aid from the American Heart Association Greater Los Angeles Affiliate. J. Ngai was supported by a Gordon Ross Foundation Predoctoral Fellowship and Y. G. Capetanaki by a Postdoctoral Fellowship from the Muscular Dystrophy Association of America. E. Lazarides is a recipient of a Research Career Development Award from the National Institutes of Health.

Received for publication 28 March 1984, and in revised form 20 April 1984.

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CHAPTER 4:

Expression of Transfected Vimentin Genes in Differentiating Murine Erythroleukemia Cells Reveals Divergent *Cis*-Acting Regulation of Avian and Mammalian Vimentin Sequences

(Submitted to *Cell*)

Expression of Transfected Vimentin Genes in Differentiating Murine Erythroleukemia Cells Reveals Divergent *Cis*-Acting Regulation of Avian and Mammalian Vimentin Sequences

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SUMMARY

We have studied the expression of transfected chicken and hamster vimentin genes in murine erythroleukemia (MEL) cells. MEL cells normally repress the levels of endogenous mouse vimentin mRNA during inducer-mediated differentiation, resulting in a subsequent loss of vimentin filaments. The expression of vimentin in differentiating MEL cells reflects the disappearance of vimentin filaments during mammalian erythropoiesis in vivo. In contrast, chicken erythroid cells express high levels of vimentin mRNA and vimentin filaments during terminal differentiation. We demonstrate here that chicken vimentin mRNA levels increase significantly in differentiating transfected MEL cells, whereas similarly transfected hamster vimentin genes are negatively regulated. In conjunction with in vitro nuclear run-on transcription experiments, these results suggest that the difference in vimentin expression in avian and mammalian erythropoiesis is due to a divergence of *cis*-linked vimentin sequences that are responsible for transcriptional and posttranscriptional regulation of vimentin gene expression. Transfected chicken vimentin genes produce functional vimentin protein and stable vimentin filaments during MEL cell differentiation, further demonstrating that the accumulation of vimentin filaments is determined by the abundance of newly synthesized vimentin.

INTRODUCTION

Intermediate filaments represent a family of cytoskeletal structures whose members are expressed in developmentally-regulated and tissue-specific patterns in vertebrate cells. Keratins are expressed in epithelial cells and cells of epithelial origin, desmin in smooth and striated muscle, glial fibrillary acidic protein in glial cells, neurofilaments in neuronal cells, and vimentin in a wide variety of both immature and differentiated cell types (reviewed by Lazarides, 1982, and Steinert et al., 1985). The specificity of intermediate filament protein expression in developing cell lineages suggests that each intermediate filament protein type plays an important morphogenetic role in cellular differentiation. An interesting facet of the study of intermediate filament proteins indeed pertains to their patterns of developmental and tissue-specific regulation. Vimentin expression is both positively and negatively regulated during the differentiation of a variety of cell types. For example, in chicken spinal cord, vimentin is replaced by neurofilament protein during the terminal differentiation of neurons (Tapscott et al., 1981). In muscle, astrocytes, and certain retinal neurons, vimentin is co-expressed with the cell type-specific intermediate filament protein (desmin, glial fibrillary acidic protein, and neurofilament protein, respectively) in the fully differentiated state (Granger and Lazarides, 1979; Tapscott et al., 1981; Yen and Fields, 1981; Schnitzer et al., 1981; Dräger, 1983). Fibroblasts, lens fibers, and avian erythrocytes express vimentin as the major intermediate filament protein subunit of the mature cell (Franke et al., 1978; Bradley et al., 1979; Ramaekers et al., 1980; Granger et al., 1982).

The generality and diversity of vimentin expression suggests that its regulation is necessarily complex. This complexity is particularly evident from a

comparison of avian and mammalian erythropoiesis. The mature avian erythrocyte is a nucleated biconvex ellipsoidally-shaped cell. Ultrastructural analyses of avian red cells have revealed a network of intermediate filaments which spans the cytoplasm, and appears to anchor the centrally located nucleus (Virtanen et al., 1979; Woodcock, 1980; Granger and Lazarides, 1982). The major subunit protein of these filaments is vimentin (Granger et al., 1982). In contrast, the anucleate, biconcave disc-shaped mammalian erythrocyte contains no intermediate filaments. Studies of human hematopoiesis in vivo have demonstrated that vimentin is expressed early in erythroid differentiation, but is lost in the erythroblastic stages (Dellagi et al., 1983).

In chicken erythroid cells, vimentin filaments are assembled rapidly and stably from a soluble pool of newly synthesized vimentin (Blikstad and Lazarides, 1983; Moon and Lazarides, 1983). The efficiency and rapidity of vimentin assembly in vivo suggests that the extent of vimentin filament formation is determined primarily by the amount of vimentin synthesized. We have shown that the expression of vimentin protein during erythropoiesis is determined primarily by mRNA abundance (Capetanaki et al., 1983; Ngai et al., 1984). In chicken embryonic erythropoiesis, vimentin mRNA is found at low levels in immature, mitotic primitive cells, and accumulates to increasingly higher levels during the terminal differentiation of the definitive erythroid lineage, apparently underlying similar changes at the protein level (Capetanaki et al., 1983). During differentiation of murine erythroleukemia (MEL) cells in vitro, vimentin mRNA levels rapidly and extensively decline (~25 fold reduction), rendering a concomitant decrease in vimentin synthesis and a subsequent loss of vimentin filaments (Ngai et al., 1984). The striking difference in the changes of vimentin mRNA abundances (and ultimately, of vimentin filaments) during mammalian and

avian erythropoiesis presents an opportunity to study not only the factors responsible for the dynamic positive and negative regulation of the vimentin gene, but also the basis for the evolutionary divergence of the two erythropoietic programs with regard to vimentin expression.

In the present study, we have addressed these issues by examining the behavior of transfected chicken and hamster vimentin genes in differentiating MEL cells. The utility of studying both resident and transfected genes during in vitro differentiation of MEL cells is well established (reviewed by Marks and Rifkind, 1978, and Chao, 1986). We demonstrate that in MEL cell lines harboring and expressing chicken vimentin genes, chicken vimentin mRNA levels either increase two to ten fold or remain constant during DMSO-mediated differentiation, whereas the resident mouse vimentin mRNA levels decline extensively (10-20 fold). Expression of mRNA from transfected hamster vimentin genes is reduced significantly in differentiating MEL cells. These results suggest that the divergence in vimentin expression in mammalian and avian erythropoiesis is due to a divergence of elements linked to vimentin sequences in *cis*. Our results further suggest the existence of general erythroid-specific trans-acting factors which have been functionally conserved from birds to mammals, and interact with chicken vimentin regulatory sequences in murine erythroid cells in a manner appropriate for chicken vimentin sequences in a chicken erythroid environment.

RESULTS

Transfection of MEL Cells with the Chicken Vimentin Gene

We constructed a plasmid, designated pAV21, specifically for the introduction of chicken vimentin gene sequences into adenine phosphoribosyl transferase (aprt) deficient cells (Figure 1a). pAV21 contains the entire 7.7 kb chicken vimentin gene coding sequence (Capetanaki et al., 1983; Zehner et al., 1987), with 2.5 kb 5' flanking and 2.5 kb 3' flanking sequences, plus a 4.0 kb hamster genomic DNA fragment containing the hamster aprt gene (Lowy et al., 1980). In this 20 kb plasmid, the hamster aprt gene resides in the same orientation as the chicken vimentin gene, and its point of RNA initiation (Nalbantoglu et al., 1986, 1987) is approximately 3.2 kb downstream from the vimentin gene's 3' terminus. pAV21 was linearized with Asp 718 and introduced into an $aprt^-$ MEL cell line, $Faprt^-585^-S$ (Deisseroth and Hendrick, 1978; Chao et al., 1983) by calcium phosphate precipitation (Wigler et al., 1979) or by poly-l-ornithine-mediated transfection (Bond and Wold, 1987). Fifty-two independent $aprt^+$ MEL cell clones were isolated and expanded for analysis. Of these $aprt^+$ transformants, 28 possessed intact chicken vimentin gene sequences, as judged by genomic DNA blotting (data not shown). Using either method of transfection, the copy numbers of chicken vimentin genes ranged from one copy to ~10-20 copies per cell (data not shown; see Table I).

Analysis of Chicken Vimentin mRNA Expression in Differentiating MEL Cells

MEL cell lines harboring intact chicken vimentin sequences either were grown in the absence of chemical inducer ("undifferentiated" cells), or were induced to differentiate by culturing in the presence of 1.8% DMSO (Friend et al., 1971) for

4-5 days, and total cellular RNA was isolated. Chicken vimentin RNA was detected by quantitative RNase protection of a homogeneously-labeled 1.9 kb ^{32}P-RNA probe specific for the first and second exons (see Figure 2b). Figure 2a, lane 2 demonstrates protection of portions of the RNA probe corresponding to the 632 nt first exon and 61 nt second exon sequences of vimentin RNA from chicken embryo fibroblasts. This probe apparently also hybridizes to mouse vimentin RNA sequences, rendering a 70 nt protected fragment (e.g., see Figure 2a, lane 3; compare with tRNA control). This 70 nt fragment is not detected in RNA from differentiated MEL cells (e.g., see Figure 2a, lane 4), and is absent from RNA preparations derived from other murine cell lines devoid of vimentin mRNA, such as the plasmacytoma MPC-11 (McTavish et al., 1983; data not shown). Ten MEL cell lines expressed correctly initiated chicken vimentin RNA at detectable levels (Figure 2a, lanes 5-24). Seven cell lines, *MEL*CV-4, *MEL*CV-5, *MEL*CV-11, *MEL*CV-26, *MEL*CV0-2, *MEL*CVO-12, and *MEL*CVO-16, induced the expression of chicken vimentin RNA 2-10 fold during differentiation (Figure 2a; see Table I). With the exception of *MEL*CVO-26, the remainder of chicken vimentin-expressing cell lines maintained relatively constant levels of chicken vimentin RNA during DMSO-mediated differentiation. *MEL*CVO-26 cells expressed chicken vimentin RNA only at very low levels, and upon differentiation, these levels decreased by approximately 2-5 fold. The variations in levels of expression and induction are typical for transfected genes in this and other systems (e.g., see Chao, 1986). As an internal control, we observe that mouse vimentin RNA levels decreased by >10-fold during differentiation of all MEL cell lines, as judged by the 70 nt fragment protected by mouse vimentin RNA sequences (Figure 2a).

Among the cell lines which induced chicken vimentin RNA during DMSO-mediated differentiation, *MEL*CV-5, *MEL*CV-26, and *MEL*CVO-12 expressed the

chicken RNA after differentiation at levels comparable to or greater than the levels of endogenous mouse vimentin RNA found in undifferentiated cells (compare the 70 nt protected fragment of mouse vimentin with the 61 nt protected fragment of chicken vimentin in lanes 5 and 6, 11 and 12, and 15 and 16). Experiments similar to the one shown in Figure 2a were performed several times, with results qualitatively similar to those shown here (data not shown).

Analysis of β-Globin RNA Levels in Transfected MEL Cells

To assess the extent of differentiation of each of the cell lines expressing chicken vimentin RNA, we analyzed the levels of β-globin RNA during differentiation of a selected subset of cell lines. A 345 nt ^{32}P-labeled RNA, complementary to mouse β^{major} globin sequences from the 5' end of the first intervening sequence (excluding the first 9 nt), to the 3' end of the second exon (excluding the last 13 nt) was synthesized and used as a probe in RNase protection experiments (Figure 3b). A 209 nt protected fragment corresponding to exon II of the β^{major} globin mRNA is induced 50-fold in the parent MEL cell line (Figure 3a, lanes 2 and 3; Table I). Our β^{major} globin probe also detects the presence of β^{minor} globin transcripts, as evidenced by the protection of an 81 nt fragment, corresponding to homology with sequences between nucleotide positions 262 to 342 of β^{major} globin (Konkel et al., 1979). The pattern shown in lanes 2 and 3 indicate that the parent MEL cell line used in this study, Faprt$^-$585$^-$S, exhibits a high ratio of β^{minor} globin to β^{major} globin RNA, and expresses a significant amount of β^{minor} globin RNA in the undifferentiated state; the induction of β^{minor} globin RNA in these cells is 3-4 fold. As a comparison, the induction pattern of β-globin RNA from another MEL cell line, Mel-F1, is shown in Figure 3a, lanes 18 and 19. This MEL cell line produces mainly β^{major} globin RNA upon differentiation with DMSO, and

comparatively little β^{minor} globin RNA in either the undifferentiated or differentiated state. Variations in β^{major} globin and β^{minor} globin ratios in different MEL cell lines have been described previously (Alter and Goff, 1978). Figure 3a, lanes 4-17 and Table I show that *MEL*CV-5, *MEL*CV-11, *MEL*CV-26, *MEL*CV-29, *MEL*CVO-2, *MEL*CVO-12, and *MEL*CVO-16 all induce β^{major} globin RNA by 10-120 fold (mean = 43 fold), and β^{minor} globin RNA by 2.5-7 fold (mean = 4 fold). Hence, the increases in chicken vimentin RNA in differentiating MEL cells are occurring in cells undergoing extensive differentiation, as judged by β-globin RNA induction.

Analysis of Hamster aprt and Mouse γ-Actin RNA Levels in Transfected MEL Cells

The specificity of induction of chicken vimentin RNA levels in differentiating MEL cells was established by assaying levels of hamster aprt RNA. If the transfected vimentin and aprt genes are independently expressed in our cell lines, then we expect to find little or no correlation in the respective steady state mRNA levels during differentiation. We also measured endogenous γ-actin RNA levels, since actin RNA levels are expected to exhibit little, if any changes during MEL cell differentiation (Ngai et al., 1984). MEL cell RNAs were hybridized to a mixture of two ^{32}P-labeled RNA probes. The hamster aprt-specific probe is a 0.9 kb RNA complementary to the 95 nt first exon and 111 nt second exon of the aprt transcript (see Figure 3d). The γ-actin-specific probe is a complementary RNA derived from human γ-actin 3' sequences, and protects an 80 nt fragment from mouse γ-actin RNA (Enoch et al., 1986). Figure 3c shows RNase protection experiments which measure simultaneously the steady state levels of aprt and mouse γ-actin RNAs. Quantitation of this gel is tabulated in Table I. A

comparison of hamster aprt RNA levels with chicken vimentin RNA levels reveals no direct correlation in either the amounts of RNA accumulated (compare Figures 2a and 3c) or in the ratios of RNAs in induced versus uninduced cells (Table I). Mouse γ-actin RNA levels vary less than two-fold during MEL cell differentiation (Table I), and the differences observed most likely reflect clonal variations of MEL cell isolates or variable levels of expression during particular DMSO inductions.

Utilization of Chicken Vimentin Gene Polyadenylation Signals

An interesting feature of the chicken vimentin gene is the occurrence and utilization of multiple polyadenylation sites (Zehner and Paterson, 1983a,b). Four consensus polyadenylation signals (5'-AATAAA-3' [Proudfoot and Brownlee, 1976]) reside 250, 298, 533, and 554 nt downstream from the translation termination codon (Zehner and Paterson, 1983a); however, only the second through fourth polyadenylation signals are utilized in the chicken tissues examined (Zehner and Paterson, 1983b), producing mRNAs of ~2.0 and ~2.3 kb in length (Zehner and Paterson, 1983a; Capetanaki et al., 1983). The utilization of chicken vimentin polyadenylation sites is tissue specific; definitive erythroid cells accumulate predominantly the 2.0 kb mRNA, whereas other tissues and cell types accumulate more equivalent amounts of the 2.0 kb and 2.3 kb mRNAs (Capetanaki et al., 1983). A comparison of hamster and chicken vimentin gene sequences at their 3' ends reveals that the mammalian vimentin gene retains only one functional signal, corresponding to the second chicken polyadenylation site (Quax et al., 1983); both hamster and mouse cells produce ~2.1 kb vimentin mRNAs (Dodemont et al., 1982; Ngai et al., 1984). We therefore were interested if the mechanism by which the second polyadenylation signal is preferentially utilized in definitive chicken

erythroid cells has been conserved in mammals. To address this question, we analyzed chicken vimentin RNA from transfected MEL cells by performing RNase protection experiments using as probe a 1 kb RNA complementary to chicken vimentin 3'-terminal sequences, from 143 nt downstream of the translation termination codon, past the last polyadenylation signal (Figure 4b). Chicken vimentin RNAs with 3' termini generated from polyadenylation signals 2, 3, and 4 protect 170, 410, and 430 nt fragments of the RNA probe (Figure 4a, lanes 3, 10 and 11). The preferential utilization of polyadenylation signal 2 in 15-day old chick embryo erythroid cells (lanes 3 and 10) as compared to chicken spinal cord (lane 11) is demonstrated in this experiment, in agreement with previous findings (Capetanaki et al., 1983). Total RNA from MEL cells expressing transfected chicken vimentin genes contains chicken vimentin RNAs with ratios of 3' termini similar to those found in chicken non-erythroid cells. *MEL*CV-26 and *MEL*CV-29 cells accumulate similar ratios of chicken vimentin RNAs in both the undifferentiated and differentiated states (lanes 4-7; compare with chicken definitive erythroid RNA [lanes 3 and 10] and chicken spinal cord poly(A)$^+$ RNA [lane 11]). All MEL cell lines that express detectable chicken vimentin RNA display 3' terminal patterns identical to those shown here for *MEL*CV-26 and *MEL*CV-29 (data not shown). Mouse fibroblastic L cells transfected with pAV21 also accumulate similar ratios of chicken vimentin transcripts as transfected MEL cells (data not shown). The RNAs exhibiting these 3' termini in MEL cells are ***bona fide*** polyadenylated transcripts, as they are greatly enriched in the poly(A)$^+$ RNA fraction (compare 10 μg total RNA from induced *MEL*CV-26 cells [lane 8] with 1 μg of poly(A)$^+$ RNA derived from the same total RNA preparation [lane 9]). From these experiments we conclude that MEL cells do not discriminate the multiple chicken vimentin polyadenylation sites in a manner specific to chicken definitive erythroid cells.

Transfected Chicken Vimentin Genes Produce Functional mRNAs and Vimentin Filaments in Differentiating MEL Cells

We examined one MEL cell line, *MEL*CV-26, for the synthesis and stabilization of vimentin protein. *MEL*CV-26 cells contain high levels of chicken vimentin RNA, which increase approximately three-fold after differentiation in the presence of DMSO (Figure 2a, lanes 11 and 12; Figure 4a, lanes 4 and 5). Cells were maintained in control medium or induced to differentiate in medium containing 1.8% DMSO for 4 days, incubated with ^{35}S-methionine for 1 hr, and fractionated with Triton X-100. Equivalent amounts of Triton X-100 insoluble protein-incorporated radioactivity were analyzed by two-dimensional electrophoresis; this material represents newly synthesized, newly assembled cytoskeletal protein (Blikstad and Lazarides, 1983; Moon and Lazarides, 1983). Figure 5 demonstrates that even though newly synthesized and assembled mouse vimentin is greatly reduced after differentiation in both the parent cell line (panels a and b) and in *MEL*CV-26 cells (panels c and d), newly synthesized and assembled chicken vimentin is not only present in undifferentiated cells (panel c), but increases in abundance upon differentiation (panel d). Liquid scintillation counting of excised gel spots reveals that chicken vimentin radioactivity increases 5-6 fold at 4 days of differentiation in *MEL*CV-26 cells, whereas mouse vimentin radioactivity decreases $\geq$4-5 fold in these cells, and 7-10 fold in the parent MEL cell line (data not shown). Cytoskeletal chicken vimentin accumulates to high steady state levels after DMSO induction of *MEL*CV-26 cells, as judged by Coomassie blue staining of the gels (panels e and f) whose autoradiograms are shown in panels c and d.

To ascertain if the cytoskeletal chicken vimentin was present in a filamentous configuration, we performed indirect immunofluorescence microscopy on undifferentiated and differentiated MEL cells, using a vimentin-specific antiserum (Granger and Lazarides, 1979). Figure 6, b and d show that vimentin filaments are lost during differentiation of the parent MEL cell line, in accordance with our previous observations using another MEL cell line, MEL-F1 (Ngai et al., 1984). When we examined *MEL*CV-26, however, we observed a striking array of intensely fluorescent vimentin filaments in both undifferentiated an differentiated cells (Figure 6, f and h). In several experiments, we have found that all *MEL*CV-26 cells are vimentin-positive, both before and after DMSO-induction, indicating that the transfected vimentin gene is expressed in all cells in the population. Since the composition of cytoskeletal vimentin in differentiated *MEL*CV-26 cells is predominantly, if not exclusively, chicken vimentin, the array shown in Figure 6h is due to the presence of chicken vimentin filaments. Hence, differentiating *MEL*CV-26 cells accumulate filamentous chicken vimentin during differentiation while simultaneously losing mouse vimentin. The results of Figures 5 and 6 have also been confirmed in another cell line, *MEL*CV-5; by both immunoblot and immunofluorescence analysis, we have found that chicken vimentin accumulates and forms filaments during differentiation of *MEL*CV-5 cells, whereas the endogenous mouse vimentin is lost (data not shown).

Expression of Hamster Vimentin Genes in Differentiating MEL Cells

The data presented thus far indicate that MEL cell lines transfected with chicken vimentin genes accumulate either increasing or constant amounts of both the chicken mRNA and protein during DMSO-mediated differentiation. However, we could not rule out the possibility that the induction (or lack of repression) of

chicken vimentin mRNA was due to aberrations of DNA transfection. We therefore transfected MEL cells with a cloned hamster vimentin gene. The plasmid construct used for this purpose, pAHV3, is analogous to pAV21, the chicken vimentin plasmid. In pAHV3 we substituted the chicken genomic sequences and ~400 bp of hamster aprt 5' flanking sequences with a 14.5 kb genomic DNA fragment containing the entire hamster vimentin gene (Quax et al., 1983) (see Figure 1). The hamster vimentin gene lies in the same orientation relative to vector and aprt gene sequences as the chicken gene does in pAV21, and is surrounded by ~3 kb 5' flanking and ~3' flanking sequences. pAHV3 DNA was linearized by Asp718 and introduced into Faprt$^-$585$^-$S cells by calcium phosphate transfection. Seven independent cell lines containing intact hamster vimentin gene sequences were obtained and used for subsequent analysis. To assay hamster vimentin RNA levels, we performed quantitative primer extension analysis, using a ^{32}P-labeled oligonucleotide complementary to hamster vimentin mRNA 37-56 nt downstream from the predicted cap site (Quax et al., 1983). Figure 7, lane 1 shows the expected 53-57 nt primer extension product using BHK-21 RNA as a source of hamster vimentin transcript (Gard et al., 1979; Tuszynski et al., 1979). Primer extension using Faprt$^-$585$^-$S RNA as template was negative (Figure 7, lanes 2 and 3). Three cell lines, *MEL*AHV-6, *MEL*AHV-9, and *MEL*AHV-12, were found to express the hamster vimentin RNA at detectable levels, and all three showed significantly reduced levels of this RNA upon differentiation (lanes 4-9). Undifferentiated *MEL*AHV-9 and *MEL*AHV-12 cells accumulated hamster vimentin RNA at ~5% of the levels found in BHK-21 cells. Upon differentiation, hamster vimentin RNA levels fell ~2-fold in *MEL*AHV-9 and 10-fold in *MEL*AHV-12 (Table II). The decrease in hamster vimentin RNA in *MEL*AHV-6 was difficult to measure, as the levels in undifferentiated cells were low, and declined below our

limits of detection in differentiated cells; we estimate the decrease to be $\geq$5-fold. Quantitation of aprt, γ-actin, and β-globin RNA levels confirmed that the decline in hamster vimentin RNA was specific, and induction of β-globin RNA was extensive (Table II).

Determination of Vimentin Gene Transcription Rates by In Vitro Nuclear Run-On Transcription Analysis

Our observation that chicken vimentin RNA increases and the murine or hamster vimentin RNA decreases during MEL cell differentiation suggests that the differences are due to *cis*-acting sequences. To establish the contribution of RNA transcription rates toward vimentin RNA accumulation levels, we performed in vitro nuclear run-on transcription analysis (McKnight and Palmiter, 1979; Groudine et al., 1981). Nuclei were isolated from MEL cells cultured in the absence or presence of DMSO for 4 days, and incubated in the presence of α-[^{32}P] UTP. ^{32}P-labeled RNA was isolated and transcription rates were determined by hybridizing with excess filter-bound DNAs. In this assay, we assume that re-initiation of transcription does not occur, and the incorporated radioactivity detected therefore reflects the polymerase density on the gene of interest (however, see Discussion). To determine the utility of this technique for our system, we performed run-on transcriptions on nuclei isolated from a MEL cell line (Mel-F1) whose endogenous vimentin, β-globin, and actin RNA accumulation kinetics have been determined previously (Ngai et al., 1984). In this cell line, steady state vimentin RNA falls to 30% of control levels at 24 hr of DMSO-induced differentiation, 10% at 48 hr, and 4% at 96 hr of differentiation; actin mRNA levels remain constant through 96 hr of differentiation, whereas β-globin mRNA increases 3-5 fold at 48 hr, and 10-20 fold at 96 hr (Ngai et al., 1984).

Figure 8a and Table III show the results of nuclear run-on transcriptions in nuclei obtained from MEL-Fl cells grown in control medium or in the presence of DMSO for 24, 48, and 96 hr. β-globin transcription increased to a maximum of 12-fold over control levels at 96 hr, and actin transcription remained fairly constant, falling to only 74% after 96 hr of differentiation. Surprisingly, murine vimentin transcription at 24 hr of differentiation decreased only slightly to 56% of control levels, and transcription of this gene was maintained at ~40% through 96 hr of differentiation. Similar results were obtained with Faprt$^-$585$^-$S cells; in two independent experiments, murine vimentin transcription decreased to only 40% of control levels at 96 hr of DMSO-induced differentiaton (Figure 8b and Table IV). Blot analysis of cytoplasmic RNA from the same cell preparations used in these experiments confirmed the extensive decline (10-20 fold) in vimentin mRNA steady state levels in both MEL-Fl and Fapart$^-$585$^-$S cells at 96 hr of differentiation (data not shown). The induction of β-globin transcription was only 3.5-5 fold in Faprt$^-$585$^-$S cells; this is due to the high level of β^{minor} globin expression in the undifferentiated state (see Figure 3a, lanes 3 and 4).

In two independent experiments using *MEL*CV-26 nuclei, transcription of the introduced chicken vimentin gene increased 2.5-4 fold (Figure 8c and Table IV), roughly paralleling the steady-state RNA levels (see Table I). Aprt and β-globin transcription both increased ~2-3 fold, approximating the increases in the respective steady-state amounts (Table I). Transcription of the mouse vimentin gene in differentiated *MEL*CV-26 cells apparently decreased to only ~70-90% of control levels. This value is an overestimate, however, as the combination of high levels of chicken vimentin transcription and limited (<10-15%), but variable cross-hybridization with the mammalian DNA sequences obscured the relatively lower mouse-specific signal. Based on these in vitro transcription experiments, we

conclude that the induction of chicken vimentin RNA in transfected MEL cells is due primarily to an increase in transcription, in contrast to a lesser change in the transcription of the endogenous vimentin gene relative to murine vimentin RNA levels. Actin transcription rates increased 2-3 fold in Faprt$^-$585$^-$S and *MEL*CV-26 cells; in separate experiments, the former accumulated ~2-fold more actin RNA during differentiation, and the latter maintained actin RNA at constant levels (Table I). As the determinations of actin transcription were only performed once, and steady-state actin mRNA was not measured within the same experiment, the observed disparity in *MEL*CV-26 actin transcription and accumulation may not be significant.

DISCUSSION

Divergence of Vimentin Expression in Avian and Mammalian Erythropoiesis Is Manifested in the Expression of Transfected Vimentin Genes in Differentiating MEL Cells

The differentiation of MEL cells in vitro has long served as a model system for mammalian erythropoiesis (Friend et al., 1971; reviewed by Marks and Rifkind, 1978). The induction of globin gene expression and stabilization of globin mRNAs in erythropoiesis have been examined intensively in this system (Ross et al., 1972; Orkin et al., 1975; Aviv et al., 1976; Orkin and Swerdlow, 1977; Nudel et al., 1977; Lowenhaupt and Lingrel, 1978; Lowenhaupt et al., 1978; Volloch and Houseman, 1981; Shaul et al., 1982). Studies of exogenous globin genes introduced into MEL cells have delineatd the *cis*-acting regulatory regions responsible for the induction of globin genes during erythropoiesis (Chao et al., 1983; Wright et al., 1983, 1984; Charnay et al., 1984, 1985). In the present study, we have undertaken MEL cell transfection experiments as an approach to understanding the molecular basis for divergent vimentin gene regulation in avian and mammalian erythropoiesis.

Two simple alternative mechanisms can explain the observed differences in vimentin regulation in avian and mammalian erythropoiesis. For both mechanisms, we presume that the expression of vimentin genes is regulated by an interaction of *trans*-acting regulatory factors with *cis*-linked sequences. In the first mechanism, a divergence in *trans*-acting regulatory factors is responsible for the differences in expression. In avian erythroid cells, the vimentin gene is either activated or derepressed by a *trans*-acting factor during terminal differentiation. In mammals, however, this activating factor either is absent, or has been replaced

or overridden by the activity of a new factor, resulting in the repression of vimentin RNA levels during erythropoiesis. If vimentin *cis*-acting regulatory sequences have been conserved, we predict that transfected chicken vimentin genes would be negatively regulated in differentiating MEL cells. In the second mechanism, divergent *cis*-acting vimentin regulatory elements are responsible for activation (or derepression) in avian erythropoiesis and repression (or de-activation) in mammalian erythropoiesis. The observed differences in gene expression therefore would be attributable to changes in the target vimentin sequence itself. This mechanism predicts that a transfected chicken vimentin gene would not be regulated in a manner appropriate for the differentiating host murine erythropoietic cell. Furthermore, if positively acting factors that activate the vimentin gene in chicken erythroid cells have been conserved in mammals as part of the general erythropoietic program (as we would expect them to be if they regulate the expression of a number of genes), then we would predict transfected chicken vimentin genes to be activated or derepressed in differentiating MEL cells.

Our results are consistent with the second mechanism described above. Chicken vimentin RNA levels either increased significantly (2-10 fold) or remained constant in the majority of chicken vimentin-expressing MEL cell lines undergoing DMSO-mediated differentiation. The increased accumulation of chicken vimentin RNA juxtaposed with the decline in mammalian vimentin RNA in differentiating MEL cells suggest that the two sequences are responding to different regulatory signals, and that this differential response is mediated by a difference in *cis*-acting regulatory sequences. The increased transcription of the chicken vimentin gene in these differentiating cells further suggests a conservation of positively-acting factors which are capable of interacting with

similarly conserved chicken vimentin regulatory elements. It appears that transfected chicken vimentin sequences respond to these putative factors in a manner appropriate for chicken vimentin sequences in an erythropoietic environment. A similar situation, in which an apparent divergence of target gene sequences is juxtaposed with a functional conservation of *trans*-acting regulatory factors, has been described for the avian and mammalian lens crystallins (Kondoh et al., 1983, 1987; Piatigorsky, 1984). Although mammalian genomes contain no δ-crystallin genes, chicken δ-crystallin genes are appropriately regulated in a lens-specific fashion when microinjected directly into murine cell nuclei (Kondoh et al., 1983) or expressed in transgenic mice (Kondoh et al., 1987). Hence, both chicken δ-crystallin and vimentin genes are appropriately expressed in murine xenogeneic environments according to the specificity of the species from which the genes originated.

The divergence of vimentin gene expression in avian and mammalian erythropoiesis, as mediated by *cis*-acting sequences, contrasts with the mechanism of fetal recruitment of embryonic globin genes in simian primates (Chada et al., 1986). In simian primates, the modern γ-globin genes exhibit a fetal pattern of expression, but are believed to have evolved from an ancestral "proto-γ" gene which exhibited an embryonic pattern of expression (Efstratiadis et al., 1980). In most non-primate mammals, the descendants of this proto-γ gene have maintained an embryonic pattern of expression. By studying the behavior of a cloned human $^{G}\gamma$-globin gene during embryogenesis of transgenic mice, Chada et al. (1986) observed an embryonic, rather than a fetal pattern of $^{G}\gamma$-globin gene expression. Their results suggested that the fetal recruitment of embryonic globin genes in simian primates is due to a temporal change in the expression of *trans*-acting factors specific for γ-like globin genes, rather than a divergence of *cis*-acting γ-

globin regulatory elements (Chada et al., 1986). Moreover, these results suggested that the *trans*-acting factors governing γ-like globin gene expression, as well as the sequences with which they interact, have been conserved from rodents to man (Chada et al., 1986).

It is formally possible that increased accumulation of chicken vimentin RNA in differentiating MEL cells is a manifestation of gene transfection. Exogenous vimentin genes may be released from appropriate regulation by virtue of their aberrant chromosomal locations (assumed to be random), the exclusion of regulatory sequences in the plasmid DNA constructs used for transfection, or the proximity of vector sequences. For example, transfected cloned α-globin genes are expressed at inappropriately high levels in non-erythroid cells (Mellon et al., 1981; Humphries et al., 1982; Treisman et al., 1983) and also appear to be maximally expressed in both undifferentiated and differentiated MEL cells (Charnay et al., 1984). In contrast, α-globin genes introduced into MEL cells by chromosome-mediated transfer are appropriately regulated during differentiation (Deisseroth and Hendrick, 1978, 1979; Deisseroth et al., 1980; Charnay et al., 1984), suggesting that chromatin configuration and/or local chromosome position effects play a key role in the regulated expression of α-globin gene expression. Several lines of evidence argue against these possibilities for transfected vimentin gene expression. Transfected hamster vimentin genes were found to be appropriately regulated. In three MEL cell lines expressing hamster vimentin genes, all exhibited significantly reduced levels of hamster vimentin RNA after DMSO-mediated differentiation. It is therefore unlikely that the observed patterns of chicken vimentin expression in differentiating MEL cells are due to the absence of regulatory influences particular to the murine vimentin chromosomal locus. The hamster vimentin gene construct used for these

transfections is similar to the construct used for the chicken gene transfections, except that the vimentin gene is surrounded by ~0.5 kb more 5' flanking and ~0.5 kb more 3' flanking sequences in the hamster gene construct, as compared to the chicken gene construct. However, we have obtained one MEL cell line containing vimentin sequences transferred on a genomic λ recombinant, λV8 (Capetanaki et al., 1983), which contains the entire chicken vimentin gene, plus 6 kb 5' flanking and 5 kb 3' flanking sequences. This cell line expresses chicken vimentin at increased levels subsequent to DMSO-induced differentiation, albeit at low levels (data not shown). We therefore believe that both vimentin plasmid constructs used in this study contain sufficient flanking sequences to confer appropriately regulated expression, and that the differences observed are not due to an artificial exclusion of regulatory elements or an interference by *cis*-linked vector or aprt sequences. Rather, the increased accumulation of chicken vimentin RNA and decreased accumulation of hamster vimentin RNA in differentiating MEL cells accurately reflect the phylogenetic class-specific differences of the transfected gene sequences. Lastly, our results from in vitro nuclear run-on transcription experiments suggest that the differences in murine and chicken vimentin RNA accumulation in differentiating MEL cells is mediated in part by posttranscriptional mechanisms (see below for further discussion), which are independent of the above mentioned transcriptional effects.

Non-Discriminatory Utilization of Chicken Vimentin Polyadenylation Signals in MEL Cells

The tissue-specific and developmentally-regulated utilization of chicken vimentin RNA 3' termini suggests an important, albeit unknown biological role for the differential expression of vimentin 3' noncoding sequences (Capetanaki et al.,

1983). We therefore examined the patterns of chicken vimentin transcript polyadenylation in transfected MEL cell lines to determine if the mechanisms responsible for the differential utilization of vimentin polyadenylation sites in chicken definitive erythroid cells have been conserved in mammalian erythropoietic cells. The data of Figure 4 demonstrate that the chicken vimentin 3' termini are not utilized in a pattern distinctive of chicken definitive erythroid cells. It is possible that our DNA construct used for transfections lacks sufficient DNA sequence information to confer erythroid-specific transcript cleavage and polyadenylation. However, all three chicken vimentin 3' termini are generated at the appropriate sites, indicating that sufficient downstream sequences indeed are present to direct proper transcript cleavage and polyadenylation (reviewed by Birnstiel et al., 1985). Our data suggest that although the factors responsible for chicken vimentin gene activation have been functionally conserved in avian and mammalian erythropoiesis, the mechanism by which the chicken erythroid-specific pattern of polyadenylation site utilization occurs has diverged.

The Role of Transcriptional and Posttranscriptional Mechanisms in the Regulation of Vimentin mRNA Levels in Differentiating MEL Cells

DMSO-mediated differentiation of MEL cells leads to a 25-fold reduction in endogenous mouse vimentin mRNA levels at 4 days of incubation (Ngai et al., 1984; this paper). In this study, we have performed in vitro nuclear run-on transcription experiments to determine the contribution of altered vimentin gene transcription rates to the changes in both endogenous and transfected vimentin mRNA steady state levels. In two untransfected MEL cell lines, we have found that transcription of the mouse vimentin gene reproducibly decreases to ~40% of control levels after 96 hr of DMSO-mediated differentiation. This ~2-fold

reduction in vimentin transcription in vitro contrasts with a corresponding 25-fold decrease in steady state vimentin mRNA. The apparent quantitative disparity in changes of vimentin transcription rates and steady state vimentin mRNA levels suggests that the decline in murine vimentin mRNA in differentiating MEL cells is due in part to posttranscriptional mechanisms. However, vimentin gene transcription in isolated nuclei may not accurately reflect the quantitative changes in transcription in vivo. Although it is widely assumed that reinitiation does not occur in these transcription reactions (e.g., Groudine et al., 1981), an overestimate of vimentin gene transcription during MEL cell differentiation could have been obtained. For example, the repression might be mediated by the induction of a labile or diffusible transcription factor, whose activity is lost during the purification of nuclei. It should be noted, however, that the disparity in the rate of transcriptional decrease in vitro (56% of control levels at 24 hr, ~40% at 48 hr, and ~40% at 96 hr) and the rate of steady state mRNA level decrease (30% of control levels at 24 hr, 10% at 48 hr, and 4% at 96 hr) argues against such a possibility. Nevertheless, direct in vivo measurements of vimentin gene transcription rates and RNA decay kinetics will be necessary to quantitate the absolute contributions of transcriptional and posttranscriptional regulation to the decline in steady state vimentin mRNA levels in differentiating MEL cells. Indeed, a combination of changes in transcription rate and posttranscriptional RNA processing, resulting in an overall greater change in mRNA steady state levels, has been demonstrated for the cell cycle regulation of cellular thymidine kinase (Stewart et al., 1987; Merrill et al., 1984; Lewis and Matkovich, 1986) and histone (Schümperli, 1986) mRNAs, as well as for the induction of hormonally-responsive RNAs (Brock and Shapiro, 1983; Robins et al., 1982; Paek and Axel, 1987). Transcriptional (Santiago et al., 1984; Farnam and Schimke, 1985) and

posttranscriptional (Leys et al., 1984) mechanisms also mediate the cell cycle regulation of dihydrofolate reductase mRNA levels.

A comparison of chicken and mouse vimentin gene transcription rates with the corresponding steady state mRNA levels in differentiating MEL cells suggests that the divergent expression of these two genes in avian and mammalian erythropoiesis is effected at both the transcriptional and posttranscriptional levels. The increase in chicken vimentin RNA levels during differentiation of one transfected cell line, *MEL*CV-26, apparently is due to an increase in the rate of transcription, as the increase in steady state chicken RNA levels approximated the increase in transcription. The data suggest that mammalian vimentin *cis*-acting sequences have diverged from chicken sequences in such a way as to respond to both a transcriptional repression (or deactivation) as well as a differentiation-induced RNA destabilization.

Expression and Persistence of Filamentous Chicken Vimentin in Differentiating MEL Cells

Our studies show that differentiating MEL cells expressing chicken vimentin RNA accumulate vimentin filaments in the absence of endogenous mouse vimentin expression. Inappropriate expression of stable intermediate filaments in cultured cells by DNA transfection or microinjection of mRNA has been reported for the keratins and for desmin (Kreis et al., 1983; Giudice and Fuchs, 1987; Quax et al., 1985). Together these results suggest that intermediate filament proteins can polymerize in vivo in the absence of accessory proteins; however, the presence of heterologous intermediate filament-associated proteins has not been ruled out in any of these cases.

We presume that chicken and mouse vimentins copolymerize when they are

coexpressed in undifferentiated cells; although our antibodies react with both chicken and mouse vimentin, and hence are not capable of discriminating between the two molecules by immunofluorescence microscopy, copolymerization of vimentin and desmin, vimentin and glial fibrillary acidic protein, and vimentin and neurofilament protein has been described (Steinert et al., 1981; Quinlan and Franke, 1982, 1983; Sharp et al., 1982; Granger and Lazarides, 1983; Quax et al., 1985). As chicken vimentin-expressing MEL cells differentiate, the composition of intermediate filaments probably changes gradually from a mouse-chicken vimentin heteropolymer to a chicken vimentin homopolymer. The accumulation of chicken vimentin filaments in differentiating MEL cells argues against the possibility that endogenous murine vimentin filaments are normally removed from the cytoplasm by accelerated protein turnover, for example by an activation of a Ca^{2+}-dependent vimentin-specific protease (Nelson and Traub, 1981, 1982b) in response to an influx of Ca^{2+} ions (Chapman, 1980; Levenson et al., 1980; Bridges et al., 1981). The functionally conserved specificity of the vimentin-desmin-specific Ca^{2+}-activated protease from fish to man (Nelson and Traub, 1982b) suggests that if such an activity were activated in differentiating MEL cells, chicken vimentin also would be degraded. Since a MEL cell line expressing low levels of chicken vimentin during differentiation also accumulates filaments in the absence of endogenous murine vimentin (data not shown), we believe that the accumulation of filaments is not due to an excess of protease substrate. Our results support a previous contention that the accumulation of vimentin filaments is determined primarily by the levels of newly-synthesized vimentin (Blikstad and Lazarides, 1983; Moon and Lazarides, 1983), which in turn are regulated at the level of mRNA abundance (Capetanaki et al., 1983; Ngai et al., 1984).

The experimentally induced expression of vimentin filaments in MEL cells

had no obvious effects on MEL cell differentiation. In the MEL cell lines described here, β-globin mRNA levels accumulated normally, and cells exhibited patterns of growth kinetics and reductions in cell volume typical of MEL cell differentiation (data not shown) (Marks and Rifkind, 1978). We have speculated previously that the removal of vimentin filaments in mammalian erythropoiesis facilitates the enucleation process (Ngai et al., 1984). The experiments described here do not address this issue, as enucleation occurs subsequent to the period of MEL cell differentiation studied, and furthermore, the maintenance of differentiating MEL cells to the anucleate reticulocytic stage is inherently problematical (Volloch and Houseman, 1981, 1982).

EXPERIMENTAL PROCEDURES

Construction of Plasmid DNAs

Manipulations of DNA were carried out according to standard protocols (Maniatis et al., 1982). For the transfection of aprt$^-$ cells with chicken vimentin sequences, a plasmid containing the entire chicken vimentin gene, with 2.5 kb 5' and 3' flanking sequences, plus the hamster aprt gene was constructed in pUC18. A 3.7 kb HincII-BamHI fragment from a λ genomic chicken vimentin recombinant, λV8 (Capetanaki et al., 1983), containing the 5' end of the gene plus 2.5 kb upstream sequences, was inserted into the polylinker region of pUC18 cleaved with HincII and BamHI. The resulting plasmid was then linearized with HincII, and this site was replaced by the ligation of XhoI linkers, yielding plasmid p5'VIMXB-1. The remaining 3' end of the vimentin gene sequences, plus 3.8 kb 3' flanking sequences contained in a 11.3 kb BamHI fragment of λV8, were ligated to the BamHI site of p5'VIMXB-1, yielding plasmid pVIMXBB. A 1.3 kb fragment at

the 3' end of the chicken genomic insert was removed by digestion with Asp718 (an isoschizomer of KpnI), and hamster aprt sequences were inserted on a 4.0 kb BglII-Asp718 DNA fragment (Lowy et al., 1980) by successive ligation of Asp718-generated cohesive ends, blunting of the remaining non-homologous Asp718 and BglII ends with DNA polymerase I large fragment, and blunt-end ligation. The final 20 kb plasmid, pAV21 (Figure 1a), typically was linearized at the single Asp718 site for introduction into mammalian cell lines.

The hamster vimentin gene was isolated on a 14.5 kb BamHI fragment (Quax et al., 1983) from a λ EMBL3 genomic library prepared from size-fractionated, BamHI-digested BHK-21 DNA (not shown). pAV21 was digested with HindIII and BamHI, BamHI linkers were ligated to the polymerase-blunted ends (corresponding to the polylinker HindIII site and hamster aprt 5' flanking BamHI site) of the 6.7 kb fragment containing hamster aprt plus pUC18, and the DNA was circularized to yield pUC-APRT. The 14.5 kb BamHI fragment carrying hamster vimentin sequences was then ligated to the BamHI ends of pUC-APRT. The resulting plasmid, pAHV3, is shown in Figure 1b.

Cell Culture and Transfections

An adherent aprt$^-$ MEL cell line, Faprt$^-$585$^-$S (Deisseroth and Hendrick, 1978; Chao et al., 1983) was maintained at 37°C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum and penicillin/streptomycin, plus 50 μg/ml 2,6-diaminopurine (DAP medium) to continually select for the aprt$^-$ phenotype. Cells were cultured in the absence of DAP ("neutral" medium) for several days prior to DNA transfection. Two methods were used to introduce DNA into MEL cells. Poly-l-ornithine-mediated transfections were carried out as described previously (Bond and Wold, 1987).

Sixteen to twenty hours prior to DNA transfection, cells were plated at a density of 1-2.5x10^6 cells per 100 mm tissue culture dish. At the time of transfection, plates were washed once with Earle's balanced salt solution (EBSS), and once with Tris-buffered saline (TBS; 25 mm Tris-Cl, 137 mM NaCl, 5 mM KCl, 1.4 mM Na_2HPO_4, 10 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.5). One milliliter of a TBS solution containing either 8 μg/ml Asp718-cleaved pAV21 DNA plus 28 μg/ml poly-l-ornithine or 4 μg/ml DNA plus 10 μg/ml poly-l-ornithine was added to each drained plate, and cells were incubated with the DNA-poly-l-ornithine solutions at 37°C for 3 hr with occasional rocking of the plates. The DNA-poly-l-ornithine solutions were removed, and the plates were washed twice with TBS. Neutral medium was then added, and the cells were allowed to recover and express aprt activity for 18 hr. $Aprt^+$ transformants were then selected by incubation with medium containing 4 μg/ml azaserine and 15 μg/ml adenine (AZA-AD medium). Individual MEL cell colonies were isolated in cloning cylinders and expanded for further analysis. The frequency of DNA transfer into $Faprt^-585^-S$ cells by this method is difficult to assess, since the concentrations of DNA and poly-l-ornithine used here are toxic to the cells, and an undetermined number of cells were lost ($\geq$50%) when plates were washed after incubation with DNA-poly-l-ornithine. Nevertheless, we have observed $aprt^+$ colonies in approximately one out of every two-three plates, yielding a transfection frequency of one $aprt^+$ transformant per initial ~4-10x10^6 cells. We also introduced DNA into $Faprt^-585^-S$ cells by calcium phosphate precipitation, as described (Wigler et al., 1979). Briefly, cells were plated in 10 ml neutral medium on 100 mm tissue culture dishes at a density of 6x10^5 cells per plate 18 hr prior to the addition of the calcium phosphate-DNA precipitate. One milliliter of precipitate containing 30 μg Asp718-cleaved pAV21 or pAHV3 DNA was added to each plate. Medium containing the calcium

phosphate-DNA precipitate was removed after a 20-22 hr incubation at 37°C, and replaced with neutral medium for 24 hr. Aprt$^+$ transformants were then selected as described above. We observed aprt$^+$ transformants at an approximate frequency of one per 2-5x10^6 cells. Cells transfected with pAV21 by the poly-l-ornithine protocol described above were designated *MEL*CVO (chicken vimentin/ornithine), whereas calcium phosphate-generated transformants were designated *MEL*CV. The hamster vimentin construct was introduced into MEL cells only by calcium phosphate precipitation, and the resulting cells were designated *MEL*AHV.

Analysis of aprt$^+$ *MEL* Transformants and Isolation of RNA

Aprt$^+$ clones were grown and tested for the presence of exogenous vimentin sequences by genomic DNA blotting (Southern, 1975), using appropriate vimentin-specific probes (data not shown). Cells containing either the entire chicken genomic portion of pAV21 or the entire 14.5 kb BamHI hamster genomic fragment of pAHV3 were studied further. Cells were adapted to growth in bacterial dishes to facilitate differentiation (Chao et al., 1983) and cultured in AZA-AD medium ("undifferentiated" cells), or induced to differentiate in AZA-AD medium plus 1.8% DMSO for 4-5 days (Friend et al., 1971). Faprt$^-$585$^-$S cells were induced to differentiate for 4 days in DAP medium plus 1.8% DMSO. Cells were harvested and total cellular RNA was prepared by the method of Chirgwin et al. (1979), as modified (Ngai et al., 1984). Spinal cord RNA from 1 week old chickens was isolated as described (Capetanaki et al., 1983), and cytoplasmic RNA was prepared from 15 day old chicken embryo erythroid cells (Moon et al., 1985). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography, essentially according to the method of Aviv and Leder (1972).

Determination of RNA Levels by Quantitative RNase Protection Mapping

Steady state mRNA levels of chicken and murine vimentin, hamster aprt, mouse β-globin and mouse γ-actin were assayed by protection of in vitro-synthesized complementary ^{32}P-labeled RNAs from RNase digestion by cellular RNAs (Zinn et al., 1983; Melton et al., 1984). Several plasmids were constructed in pT7SP6 (Axelrod and Kramer, 1985) for the synthesis of ^{32}P-RNA probes (see below). For the detection of chicken vimentin 5' sequences, the 3.7 kb HincII-BamHI fragment of λV8, containing the first and second exons (see Figure 1a) was inserted into the respective polylinker sites in pT7SP6. For probe synthesis, the template was cleaved with HindIII, and a 1.9 kb ^{32}P-labeled complementary RNA was generated, using T7 phage polymerase (see Figure 2b). A 1 kb chicken vimentin 3'-specific probe was synthesized from a template consisting of 3' untranslated sequences 143 nt downstream of the translation termination codon through 0.6 kb of 3' flanking sequences (Figure 4b). For β-globin RNA analysis, the 5' 1.9 kb EcoRI-BamHI fragment from the mouse β^{major} β-globin genomic insert of pMB9-βG2 (Konkel et al., 1978) was subcloned (pβ5'). A BstN1-cleaved pβ5' template was transcribed with SP6 phage polymerase, yielding ^{32}P-labeled RNA complementary to IVS I and exon II (Figure 3b). A hamster aprt-specific probe was synthesized using SP6 phage polymerase from a BamHI-cleaved recombinant containing a 0.9 kb hamster aprt fragment (p5'APRT, Figure 3d). This probe is protected by the first and second exons of aprt, and also contains IVS I and a portion of IVS II sequences (Nalbantoglu et al., 1986, 1987). Mouse γ-actin RNA was detected using a probe synthesized from a human γ-actin template cloned in pSP64 (Enoch et al., 1986).

^{32}P-labeled complementary RNA probes were synthesized with SP6 or T7

phage polymerases, using the above-mentioned templates, essentially under conditons described by Green et al. (1983) and Zinn et al. (1983). α-[^{32}P]CTP (3000 Ci/mmole) was diluted with unlabeled CTP and included in the transcription reactions at the following final concentrations and specific activities: 50 μM at 200 Ci/mmole for 5' vimentin, 50 μM at 50 Ci/mmole for β-globin, and 100 μM at 100 Ci/mmole for aprt, γ-actin, and 3'-vimentin.

Typically, $1x10^5$ - $2x10^5$ cpm ^{32}P-RNA was hybridized to 10 μg MEL cell total RNA at 45°C for 14-17 hr in 30 μl 80% formamide, 40 mM PIPES, 400 mM NaCl, 1 mM EDTA, pH 6.4. The hybridization reactions were then diluted with 300 μl 20 mM Tris-Cl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 50 μg/ml RNase A, 900 U/ml RNase T_1, and incubated at 30°C for 1 hr. Ten microliters of 20% SDS and 100 μg of Proteinase K were added, and samples were digested at 37°C for 30-60 min. Following phenol extraction, 10 μg carrier beef liver tRNA was added, and RNA was precipitated with ethanol. Protected ^{32}P-RNA fragments were resolved on 0.25 mm-thick, 5% polyacrylamide/8 M urea denaturing gels (Maxam and Gilbert, 1980) and exposed to Kodak XAR-5 film with intensifying screens.

For quantitation, ^{32}P-radioactivity in gel slices was assayed by Cerenkov radiation, as detected in the ^{3}H channel of a Beckman 1801 liquid scintillation counter; each sample was counted for 20 min. Liquid scintillation counting of gel slices immersed in fluor gave similar results. The following gel slices were excised for quantitation: the 632 nt exon I fragment for chicken vimentin; the 209 nt and 81 nt fragments for β^{major} and β^{minor} globins, respectively; the 111 nt exon II fragment for hamster aprt; and the 80 nt fragment for mouse γ-actin. Radioactivity in gel slices was directly proportional to the amount of RNA hybridized, as determined by serial dilution of cellular RNAs (data not shown). In cases where radioactivity was too low to be determined accurately (less than 2 σ

above background + 2 σ), autoradiograms using pre-flashed film (Laskey and Mills, 1975) were scanned with a densitometer, and peak areas were integrated. The data of Table I were derived from the gels shown in Figures 2 and 3. Results qualitatively similar to the ones shown here were obtained in separate, multiple determinations (data not shown).

Metabolic Labeling with [^{35}S]-Methionine and Immunofluorescence Microscopy

MEL cells cultured in the absence or presence of 1.8% DMSO for 4 days were labeled with [^{35}S]-methionine for 1 hr as described previously (Ngai et al., 1984). Equivalent amounts of protein-incorporated [^{35}S] radioactivity in Triton X-100-insoluble residues from each sample (Blikstad and Lazarides, 1983; Moon and Lazarides, 1983) were loaded on two-dimensional isoelectric focusing/SDS-polyacrylamide gels (O'Farrell, 1975); gels were stained with Coomassie blue to visualize total protein, and then impregnated with 2,5-diphenyloxazole for fluorography (Bouves and Laskey, 1974). Immunofluorescence microscopy using an anti-chicken vimentin antiserum (Granger and Lazarides, 1979) was performed exactly as described previously (Ngai et al., 1984).

Detection of Hamster Vimentin mRNA by Quantitative Primer Extension Analysis

An oligonucleotide with the sequence 5'-TGCGAACCGCGGGAGTGCTG-3' (synthesized at the Caltech Microchemical Facility) was labeled with T4 polynucleotide kinase in the presence of γ-[^{32}P]-ATP and used for primer extension analysis, essentially as described by Jones et al. (1985). This oligonucleotide is complementary to hamster vimentin mRNA in the 5' non-coding region, between positions 37 through 56 relative to the RNA initation site (Quax et al., 1983). One-tenth of a picomole ^{32}P-labeled oligonucleotide primer was hybridized to total cellular RNA for 2 hr at 55°C in 10 µl 10 mM Tris-Cl, pH 8, 250 mM NaCl, 2 mM EDTA. Samples were diluted with 25 µl 50 mM Tris-Cl, pH 8.7, 10 mM $MgCl_2$, 5 mM DTT, 350 µM dATP, TTP, dCTP, dGTP, 10 µg/ml actinomycin D, 600 units/ml RNasin, 330 U/ml AMV reverse transcriptase (FPLC-purified, Pharmacia), and incubated for 1 hr at 42°C. Following phenol extraction and ethanol precipitation, extension products were separated on 0.25 mm-thick 8%

polyacrylamide/8 M urea gels and visualized by exposure to Kodak XAR-5 film in the presence of an intensifying screen. The 53-57 nt extension products, corresponding to correctly initiated hamster vimentin mRNA, were quanitated by Cerenkov radiation determination of excised gel slices. Serial dilution of BHK-21 RNA (a source of high levels of hamster vimentin RNA) over a 20-fold range yielded a commensurate decrease in radioactive extension products (data not shown). Tripling the amount of ^{32}P-labeled oligonucleotide primer in hybridizations had no effect on the amount of extension product synthesized (data not shown).

In Vitro Nuclear Run-On Transcriptions

Transcription in isolated nuclei was performed by an amalgam of several published procedures (McKnight and Palmiter, 1979; Groudine et al., 1981; Greenburg and Ziff, 1984; Linial et al., 1985; Carey et al., 1986). MEL cells were cultured for up to 4 days in the presence or absence of 1.8% DMSO. Cells were washed several times in phosphate-buffered saline, and lysed in ice cold reticulocyte standard buffer (RSB: 10 mM Tris-Cl, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.4) plus 0.5% NP-40, 1 mM DTT, and 50-100 units/ml RNasin. All subsequent steps were carried out at 0°C, unless otherwise noted. Nuclei were pelleted by centrifugation at 1000 xg for 4 min, and post-nuclear supernatants were saved for preparation of cytoplasmic RNA by phenol:chloroform extraction. Nuclear pellets were rinsed gently in RSB alone, repelleted and resuspended in nuclear freezing buffer (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% (w/v) glycerol) at an approximate density of 10^7 nuclei per 100 µl. Nuclei either were frozen and stored at -70°C and thawed prior to use (Experiment I), or used immediately (Experiment II and MEL-F1 transcriptions).

Each transcription reaction was carried out with ~10^7 nuclei. Nuclei were preincubated at 0°C for 5-10 min in nuclear freezing buffer, pelleted for 8-10 sec in a variable speed Eppendorf microcentrifuge (setting 5), and resuspended in 50 µl nuclear freezing buffer. Fifty microliters of 2X run-on buffer (10 mM Tris-Cl, pH 8, 300 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 1 mM each ATP, CTP, and GTP) containing 320 µCi α-[^{32}P]UTP (~3000 Ci/mmol, dried down to a volume of ~5 µl) was added to each sample, and transcription was allowed to proceed for 15 min at 30°C. We have determined that incorporation of α-[^{32}P]UTP into RNA is linear for this duration at 30°C, in both uninduced and induced nuclei (data not shown). Nuclei were iced, pelleted, resuspended in 200 µl 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, plus 40 µg carrier beef liver tRNA, and high molecular DNA was digested with 75 units DNase (FPLC-purified, Pharmacia) at 30°C for 15 min. Samples were digested by the addition of 10 µl 20% SDS, 100 µg Proteinase K and incubation at 42°C for 45-60 min. RNA was isolated by extraction with 300 µl phenol, back-extraction of the organic phase with 100 µl 10 mM Tris-Cl, pH 8, 1 mM EDTA (TE), pooling the aqueous phases, and re-extraction with 400 µl phenol:SEVAG (1:1; SEVAG is chloroform:isoamyl/alcohol, 24:1). Samples were adjusted to 0.2 M NH_4OAc, and RNA was precipitated with 2 volumes of ethanol, and washed once with 80% ethanol. RNA was resuspended in 50 µl TE, and RNA was separated from unincorporated α-[^{32}P]UTP by chromatography on 1 ml Sephadex G-50 (medium) spin columns. Within a given experiment, equivalent amounts of radioactivity ($3.5x10^6$ cpm in Experiment I, $7.0x10^6$ cpm in Experiment II, and $1x10^7$ cpm in MEL-F1 transcriptions) were hybridized to nitrocellulose filters in 1 ml of solution.

Plasmid DNAs were linearized and immobilized on nitrocellulose according to Kafatos et al. (1979), using a Schleicher and Schuell dot blot apparatus. Ten micrograms of each plasmid was applied to each dot. The plasmids used were: pVIMXBB for detection of chicken vimentin gene transcription; pSThvim, a pT7SP6 recombinant containing the 14.5 kb BamHI hamster vimentin genomic fragment, for mouse vimentin; pβ5' for β-globin; pSP64γ-actin for mouse γ-actin, p5'APRT for hamster aprt, and pT7SP6 as a negative control. Filters were prehybridized at 65°C for 2 hr in 20 mM Tris-Cl, pH 7.5, 10 mM EDTA, 0.3 M NaCl, 0.2% SDS (2X TESS), plus 0.25 mg/ml beef liver tRNA and 1X Denhardt's solution (Denhardt, 1966). ^{32}P-labeled RNA in TE was heated to 65°C for 2-3 min, iced, and added to 1 ml 2X TESS, 0.1 mg/ml beef liver tRNA, 1X Denhardt's solution preheated to 65°C, and hybridized in microcentrifuge tubes at 65°C for 45 hr (Experiment I), 61 hr (Experiment II), or 66 hr (MEL-F1 transcriptions). Filters were washed three times with 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) at 65°C for 30 min each, once with 2X SSC at room temperature, and digested with 5 μg/ml RNase A and 25 units/ml RNase T_1 in 2X SSC at 37°C for 30 min. Filters were washed finally in 3-4 changes of 2X SSC, 0.2% SDS at 65°C for 30 min each, air-dried, and exposed to preflashed Kodak XAR-5 film with intensifying screens. Radioactivity in each dot was quantitated by Cerenkov radiation, as described above. For each individual filter, the radioactivity in the pT7SP6 dot (18-20 cpm, less than 1-2 cpm over machine background) was subtracted from the gross radioactivity of each of the other dots. Dilution of input radioactivity in the hybridization yielded comparable decreases in hybridized RNA. The inclusion of 2 μg/ml α-amanitin in nuclear pre-incubations and transcriptions abolished all detectable radioactivity bound to the above-described plasmid dots, indicating that the transcription as assayed by our

plasmids was due to RNA polymerase II activity (Kedinger et al., 1979; Lindell et al., 1970).

Acknowledgements

We thank T. Enoch and T. Maniatis (Harvard University) for their gift of pSP64-γ-actin, L. Brunet (University of California at Los Angeles) for helpful suggestions on in vitro nuclear transcription experiments, and Z. Zehner (Medical College of Virginia) for graciously providing chicken vimentin gene sequence data prior to their publication. This work was supported by a grant to E.L. from the Aging Institute of the National Institutes of Health. J.N. was also supported by a Gordon Ross Medical Foundation predoctoral fellowship, and V.C.B. by an N.I.H. postdoctoral fellowship.

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FIGURE LEGENDS

Figure 1. Plasmid DNA constructs used for transfection of MEL cells. a) Structure of pAV21, a chicken vimentin-hamster aprt plasmid. b) Structure of pAHV3, a hamster vimentin-hamster aprt plasmid. Solid bars indicate gene coding regions. Open bars indicate vimentin flanking sequences, whereas hatched bars show aprt flanking sequences.

Figure 2. Analysis of chicken vimentin RNA levels in undifferentiated and differentiated transfected MEL cells. a) A ^{32}P-labeled RNA probe complementary to the first and second exons of chicken vimentin mRNA (panel b) was hybridized to cellular RNAs and subjected to RNase digestion as described in Experimental Procedures. Protected fragments were resolved on denaturing polyacrylamide gels. *M*, ^{32}P-labeled pBR322-HpaII DNA markers. *tRNA*, protection of probe from RNase digestion after hybridization with 10 μg tRNA. *CEF*, protection of 2 μg chick embryo fibroblast total RNA. Fragments corresponding to the 632 nt first exon and 61 nt second exon are protected. *Lanes* 3-24, protection from 10 μg MEL cell RNA, from cells cultured in the absence (-) or presence (+) of 1.8% DMSO for 4-5 days. Note the protection of a 70 nt fragment by mouse vimentin RNA. The upper portion of lanes 1-18 was exposed for 1.5 days. To facilitate visualization of 60-70 nt fragments (~10-fold lower specific activity than the 632 nt exon I fragment), the lower portion of the same gel was exposed for 4.5 days. Lanes 19-24 were exposed for 4 days. *Lower panel*, the upper region of lanes 1-18 was exposed for 4.5 days to show lower intensity bands.

Figure 3. Analysis of β-globin, hamster aprt, and mouse γ-actin RNA levels in MEL cells. *a*) β-globin RNA analysis. A ^{32}P-labeled RNA probe for mouse β^{major} globin (panel b) was used for RNase protection and electrophoretic analysis as described in Experimental Procedures and the legend of Figure 2. The β^{major} globin probe is protected by both β^{major} globin mRNA (209 nt fragment), as well as by β^{minor} globin mRNA (81 nt fragment). *M*, ^{32}P-labeled pBR322-HpaII markers. *tRNA*, tRNA (10 μg) control. *Lanes* 2-19, protection from 10 μg RNA isolated from uninduced (-) and induced (+) MEL cells. The RNAs used in this assay, as well as the assay in panel c, were from the same preparations as were used for the experiments shown in Figure 2. *c*) Hamster aprt and mouse γ-actin RNA analysis. A mixed ^{32}P-labeled RNA probe consisting of a complementary γ-actin RNA (Enoch et al., 1986) and a complementary hamster aprt RNA (panel d) was used, as described above. Protection of the aprt probe generates fragments of 111 nt (exon II) and 95 nt (exon I); protection of the γ-actin probe yields an 80 nt fragment. *M*, pBR322-HpaII markers. *tRNA*, protection of mixed probe after hybridization with 10 μg tRNA. *Lanes* 2 and 3, protection of 10 μg *MEL*CVO-16 (induced) RNA with the aprt probe only or actin probe only, respectively. *Lanes* 4-19, protection of the mixed probe with 10 μg MEL cell RNA, from DMSO-induced (+) or uninduced (-) cells.

Figure 4. Analysis of chicken vimentin RNA 3' termini. The utilization of chicken vimentin RNA 3' termini was determined by protection of the ^{32}P-labeled RNA probe shown in panel b. The positions of the consensus polyadenylation signal (5'-AATAAA-3') are indicated (Zehner and Paterson, 1983a). The sites are numbered 1 through 4, in a 5' to 3' direction. The expected sites of 3' termini, together with the predicted sizes of protected probe fragments (170, 410, 430 nt)

are shown. a) Protected probe fragments resolved on a denaturing polyacrylamide gel. *M*, pBR322-HpaII markers. *tRNA* and *Faprt⁻585⁻S*, negative controls showing probe protection after hybridization with 10 µg tRNA or 10 µg Faprt⁻585⁻S (uninduced) RNA. Lane 3, protection by 2 µg 15 day-old chick embryo erythroid total cytoplasmic RNA. *Lanes* 4-7, protection using 10 µg *MEL*CV-26 and *MEL*CV-29 RNA, from uninduced cells (-) or cells induced with 1.8% DMSO for 4 days (+). *Lanes* 8 and 9, protection by 10 µg total RNA and 1 µg poly(A)$^+$ RNA, respectively, from *MEL*CV-26 cells cultured in 1.8% DMSO for 3 days. *Lanes* 10 and 11 show protection patterns using 1 µg 15 day-old chick embryo erythroid cytoplasmic poly(A)$^+$ RNA, and 1 µg 1 week-old chick spinal cord poly(A)$^+$ RNA, respectively. Lanes 1-7 were exposed for 48 hr and lanes 8-11 were exposed for 7 hr.

Figure 5. Analysis of newly assembled vimentin by two-dimensional electrophoresis. MEL cells were grown in the absence or presence of 1.8% DMSO for 4 days, labeled for 1 hr with [^{35}S] methionine, and fractionated with Triton X-100. Equivalent amounts of protein-incorporated radioactivity from insoluble residues were electrophoresed and fluorographed (panels a-d). *a*) Uninduced parental Faprt⁻585⁻S MEL cells. *b*) Induced Faprt⁻585⁻S cells. *c*) Uninduced *MEL*CV-26 cells. *d*) Induced *MEL*CV-26 cells. Migrations of mouse vimentin (m) and chicken vimentin (c) are indicated. *e*) and *f*), portions of Coomassie blue stained gels whose fluorographs are shown in panels c and d, respectively.

Figure 6. Vimentin-specific immunofluorescence microscopy. MEL cells were cultured in the absence (panels a, b, e, and f) or presence (panels c, d, g, and h) of 1.8% DMSO for 4 days and prepared for immunofluorescence microscopy using a

vimentin-specific antibody. Cells were visualized with phase contrast (*a*, *c*, *e*, *g*) and epifluorescence (*b*, *d*, *f*, *h*) optics. Vimentin filaments in parental Faprt$^-$585$^-$S cells (panel b) are lost subsequent to differentiation (panel d). *MEL*CV-26 cells contain vimentin filaments both before (panel f) and after (h) DMSO-mediated differentiation. Bar, 10 μm.

Figure 7. Quantitative primer extension analysis of hamster vimentin RNA. Extension products generated from a hamster vimentin-specific ^{32}P-labeled oligonucleotide primer using 5 μg total cellular RNA templates were resolved on an 8% polyacrylamide/8 M urea gel. One-half of each sample was loaded on the gel. The position of 54-57 nt products, corresponding to correctly initiated hamster vimentin mRNA, is indicated. *M*, pBR322-HpaII markers. *Lane* 1, BHK-21 RNA. *Lanes* 2-9, MEL cell line RNAs, from cells grown in the absence (-) or presence (+) of DMSO for 4 days. Lane 1 was exposed for 1 day; lanes 2-9 were exposed for 7 days. The radioactivity in the extension products in lanes 4 or 6 represent ~5% of the radioactivity in the products in lane 1, as determined by Cerenkov radiation of excised gel slices (data not shown).

Figure 8. In vitro nuclear run-on transcription analysis. Plasmid DNA dots immobilized to nitrocellulose were hybridized to ^{32}P-labeled RNA synthesized in isolated MEL cell nuclei, washed, and exposed to x-ray film. V_m, detection of endogenous mouse vimentin gene transcription with pSThvim, a hamster vimentin DNA plasmid. A strong signal is readily obtained, due to the high sequence homology among mammalian vimentin sequences (Ferrari et al., 1986). β, β-globin; *Ac*, actin; V_C, chicken vimentin; *aprt*, hamster aprt; (-), pT7SP6 vector DNA. *a*) Transcription in isolated nuclei from MEL-Fl cells cultured in the

absence or presence of 1.8% DMSO, as indicated. *b*) and *c*), Transcription in nuclei from parental Faprt$^-$585$^-$S cells or *MEL*CV-26 cells (respectively) grown in the absence or presence of DMSO for 4 days. The data of panels b and c are from Experiment II (see text).

Table I. Quantitation of Steady State RNA Levels in Undifferentiated and Differentiated *MEL*CV and *MEL*CVO Cells

		Steady State RNA Ratios (Differentiated Levels/Undifferentiated Levels)				
Cell Line	Approximate Number of Transfected Genes/Cell[a]	Chicken Vimentin	Hamster APRT[b]	Mouse β^{major} Globin[b]	Mouse β^{minor} Globin[b]	Mouse γ-Actin[b]
MEL Faprt⁻585⁻S	0	-	-	50	3.7	1.7
*MEL*CV-5	10-20	4[b]	1.0	9.1	2.5	0.7
*MEL*CV-11	10-20	2[c]	1.2	39	3.7	1.0
*MEL*CV-26	1	2.9[b]	1.9	35	2.9	0.9
*MEL*CV-29	10-20	1.2[b]	1.2	35	3.8	1.2
*MEL*CVO-2	2-4	≥10[c]	1.6	42	3.4	1.0
*MEL*CVO-12	1	9[b]	1.3	120	7.2	0.7
*MEL*CVO-16	2-4	≥5-10[c]	3.2	40	3.8	0.5
*MEL*CV-4	1	3[c]	ND[d]	ND[d]	ND[d]	ND[d]
*MEL*CVO-23	1	1[c]	ND[d]	ND[d]	ND[d]	ND[d]
*MEL*CVO-26	1	≤0.2-0.5[c]	ND[d]	ND[d]	ND[d]	ND[d]

[a]Estimated from genomic DNA blots (data not shown).

[b]Determined by Cerenkov radiation in excised gel slices.

[c]Determined by densitometry of autoradiograms.

[d]ND = not determined.

Table II. Quantitation of Steady State RNA Levels in Undifferentiated and Differentiated *MEL*AHV Cells

		Steady State RNA Ratios (Differentiated Levels/Undifferentiated Levels)				
Cell Line	Approximate Number of Transfected Genes/Cell[a]	Hamster Vimentin	Hamster APRT[b]	Mouse β^{major}Globin[b]	Mouse β^{minor}Globin[b]	Mouse γ-Actin[b]
*MEL*AHV-6	2-4	est. <0.2[c]	0.6	48	4.0	0.6
*MEL*AHV-9	1-2	0.4[b]	1.0	11	2.7	1.4
*MEL*AHV-12	4-6	0.1[b]	0.7	17	3.8	1.5

[a]Estimated from genomic DNA blots (data not shown).

[b]Determined by Cerenkov radiation in excised gel slices (gels not shown for β-globin, aprt, and actin determinations).

[c]See text.

Table III. Transcription in Nuclei Isolated from MEL-F1 Cells

	Mouse Vimentin		β-Globin		Actin	
Cells	cpm[a]	Fraction of Control	cpm[a]	Fraction of Control	cpm[a]	Fraction of Control
Control (-DMSO)	83	(1.0)	20	(1.0)	142	(1.0)
24 hr DMSO	47	0.56	42	2.1	114	0.80
48 hr DMSO	32	0.39	116	5.8	110	0.78
96 hr DMSO	35	0.42	242	12	104	0.74

[a]Net Cerenkov radiation, with background hybridization to pT7SP6 filter dots (18-20 cpm) subtracted.

Table IV. Transcription in Nuclei Isolated from Faprt⁻585⁻S and *MEL*CV-26 Cells

	Experiment I			Experiment II		
	cpm,[a] Undifferentiated	cpm,[a] Differentiated[f]	Differentiated/ Undifferentiated	cpm,[a] Undifferentiated	cpm,[a] Differentiated[f]	Differentiated/ Undifferentiated
Faprt⁻585⁻S						
Mouse vimentin	27	11	0.41	30	14	0.46
β-globin	6	30	5.0	14	49	3.5
Actin	ND[b]	ND[b]	ND[b]	22	51	2.3
*MEL*CV-26						
Chicken vimentin	28	120	4.2	182; 176[d]	430; 468[d]	2.5[e]
β-globin	7	21	3.1	18; 16[d]	46; 46[d]	2.7[e]
Hamster aprt	10	30	2.9	30	58	1.9
Actin	ND[b]	ND[b]	ND[b]	9	35	3.9
Mouse vimentin	33	24	0.72[c]	50	44	0.87[c]

[a]Net Cerenkov radiation, with background hybridization to pT7SP6 filter dots (18-20 cpm) subtracted.

[b]ND = not determined.

[c]The ratios given here for mouse vimentin transcription are overestimated, due to crosshybridization with chicken vimentin transcripts (see text).

[d]Radioactivity in duplicate filters from duplicate hybridizations.

[e]These ratios reflect the averages of the duplicate determinations.

[f]Nuclei from cells cultured in DMSO for 96 hr.

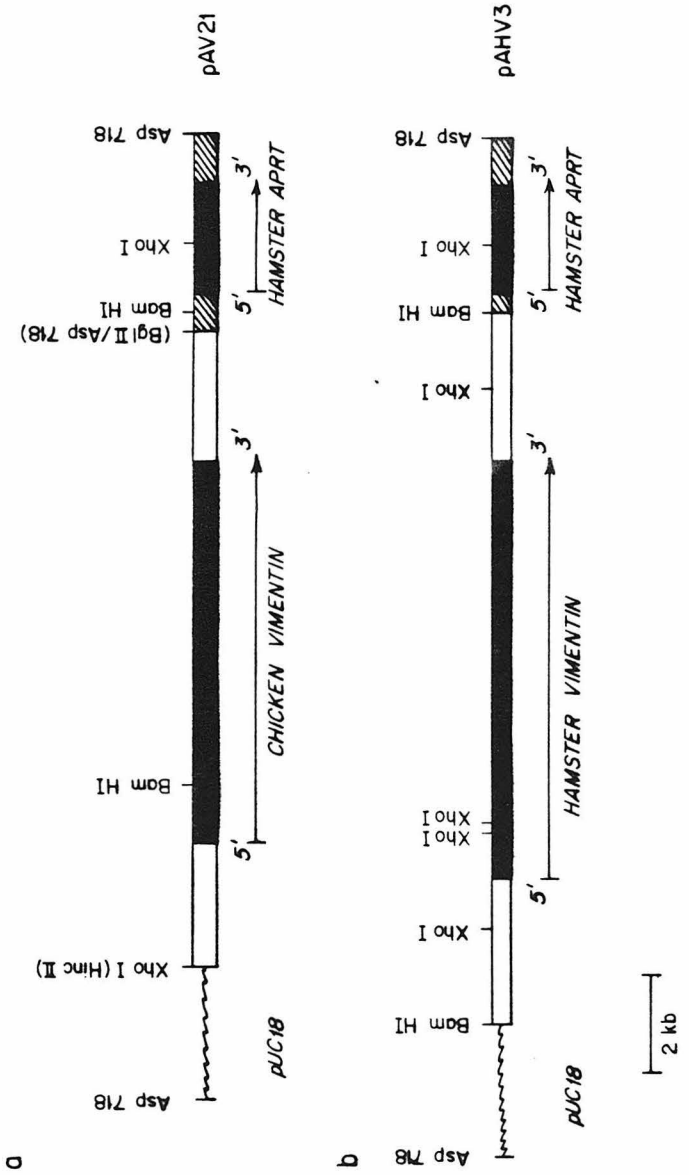
a
pAV21
Asp 718
Xho I
Bam HI
(BglII/Asp 718)
Xho I (HincII)
Asp 718
3'
5'
HAMSTER APRT
3'
5'
CHICKEN VIMENTIN
pUC18
b
pAHV3
Asp 718
Xho I
Bam HI
Xho I
Xho I
Bam HI
Asp 718
3'
5'
HAMSTER APRT
3'
5'
HAMSTER VIMENTIN
pUC18
2 kb

Figure 1

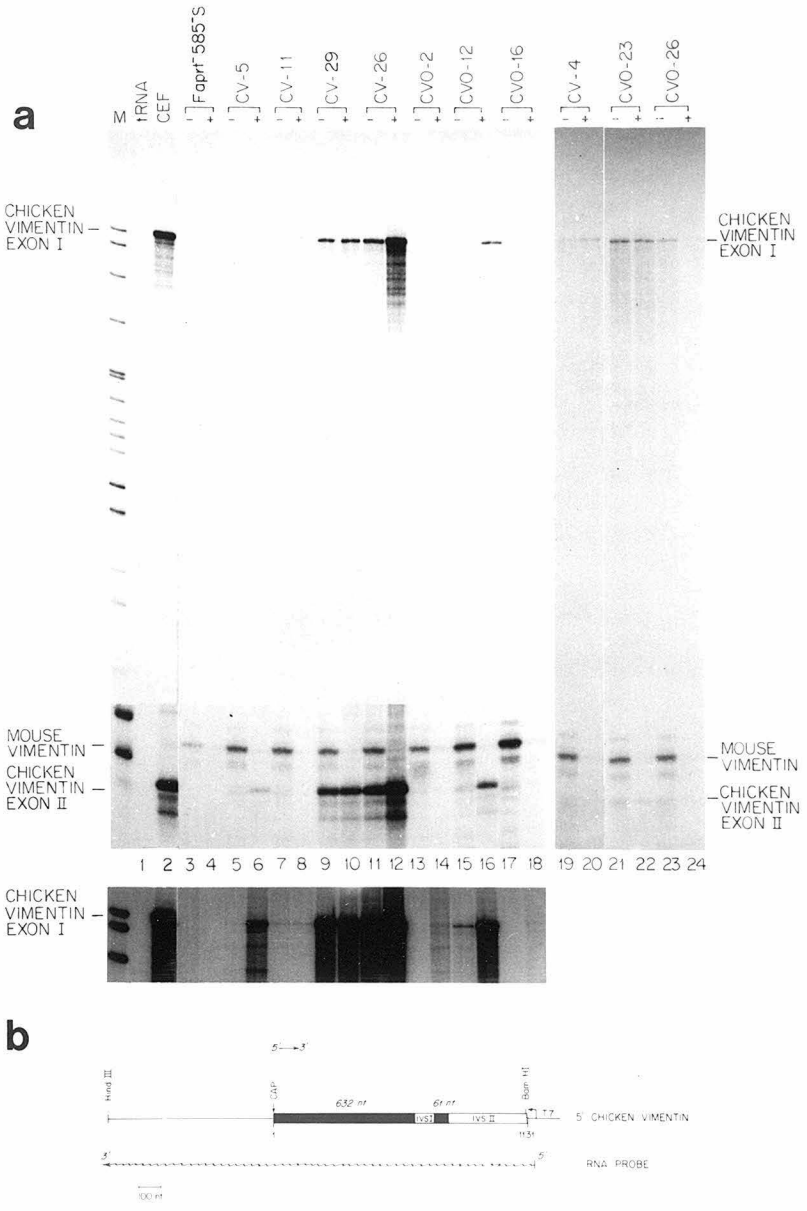
a
M
tRNA
CEF
Fαprf 585 S
CV-5
CV-11
CV-29
CV-26
CVO-2
CVO-12
CVO-16
CV-4
CVO-23
CVO-26
CHICKEN VIMENTIN EXON I
MOUSE VIMENTIN
CHICKEN VIMENTIN EXON II
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
b
Hind III
CAP
632 nt
61 nt
IVS I
IVS II
T7
Bam HI
5' CHICKEN VIMENTIN
RNA PROBE
100 nt

Figure 2

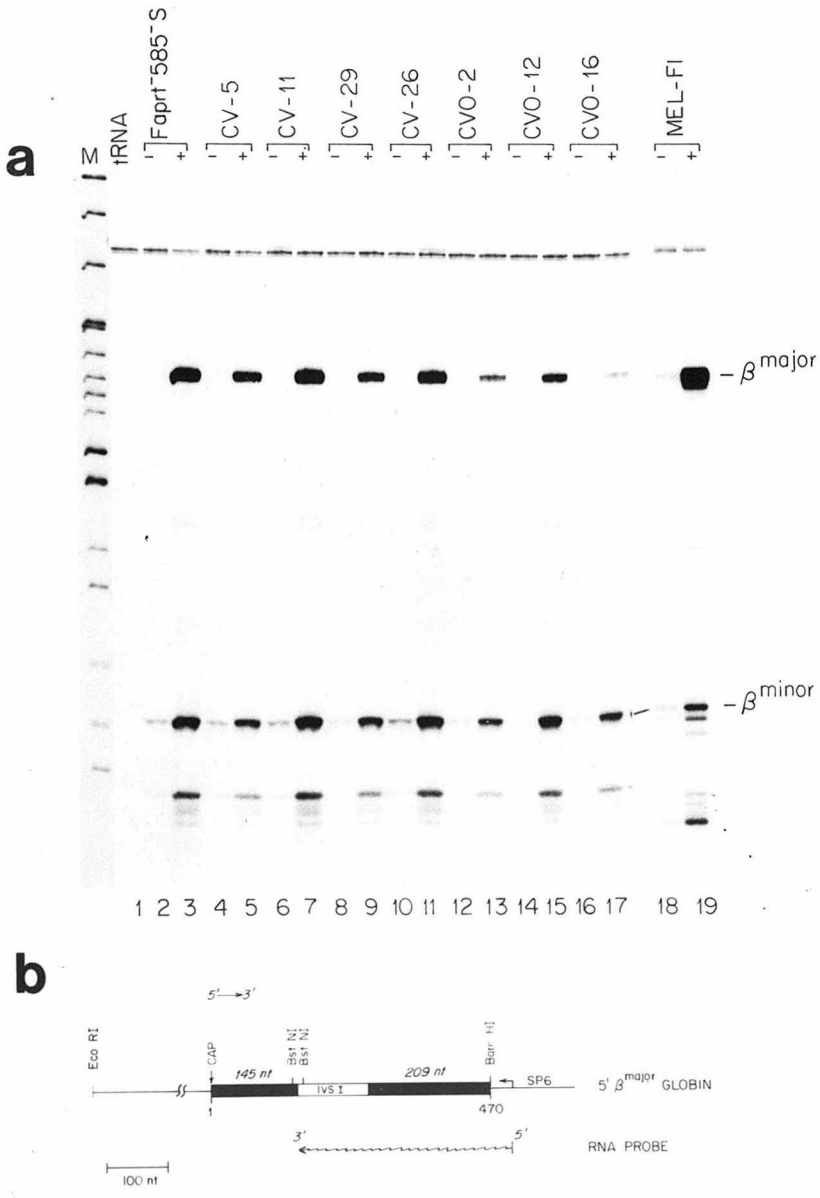
a
M
tRNA
Faprt-585-S
CV-5
CV-11
CV-29
CV-26
CVO-2
CVO-12
CVO-16
MEL-FI
- β major
- β minor
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
b
5'→3'
Eco RI
CAP
Bst NI
Bst NI
145 nt
IVS I
209 nt
Bam HI
SP6
5' β major GLOBIN
1
470
3'
5'
RNA PROBE
100 nt

Figure 3a,b

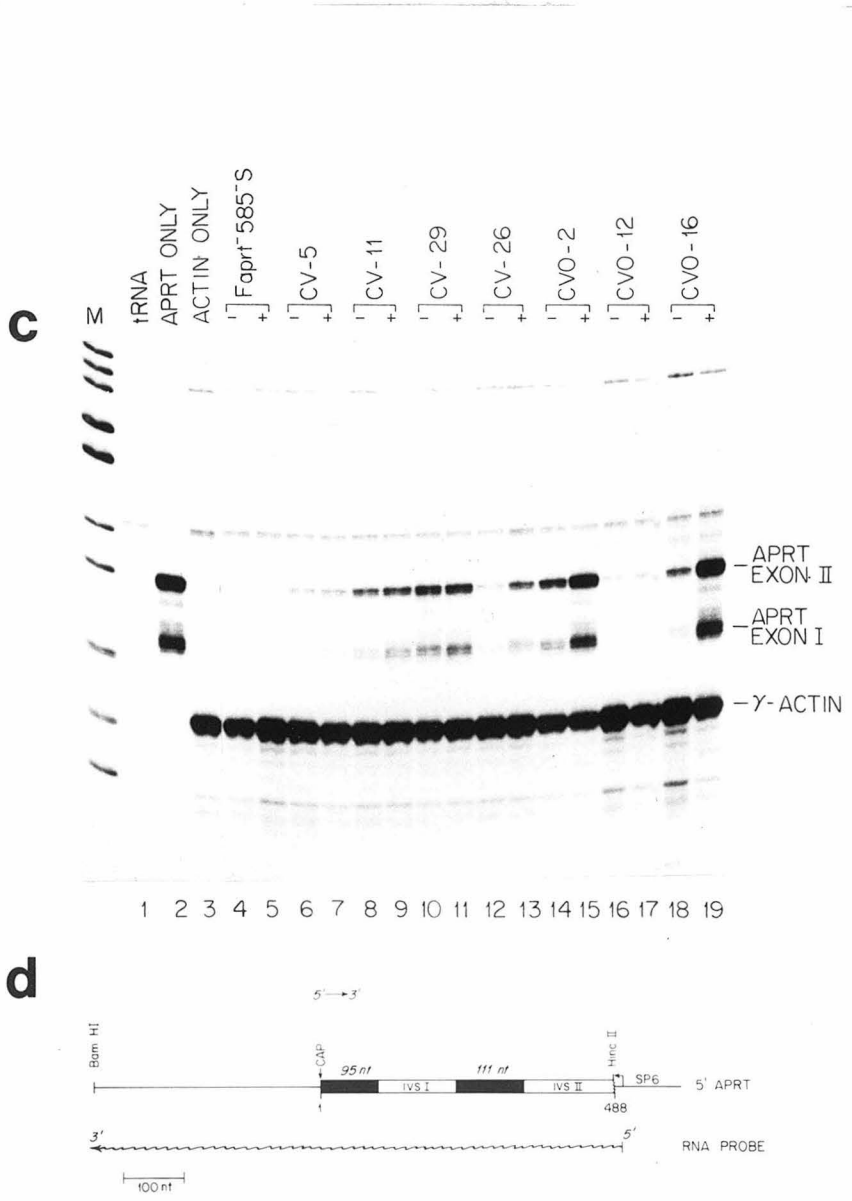
c
M
tRNA
APRT ONLY
ACTIN ONLY
Faprt⁻585⁻S
CV-5
CV-11
CV-29
CV-26
CVO-2
CVO-12
CVO-16
APRT EXON II
APRT EXON I
γ-ACTIN
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
d
5'→3'
Bam HI
CAP
95 nt
111 nt
Hinc II
SP6
5' APRT
IVS I
IVS II
1
488
3'
5'
RNA PROBE
100 nt

Figure 3c,d

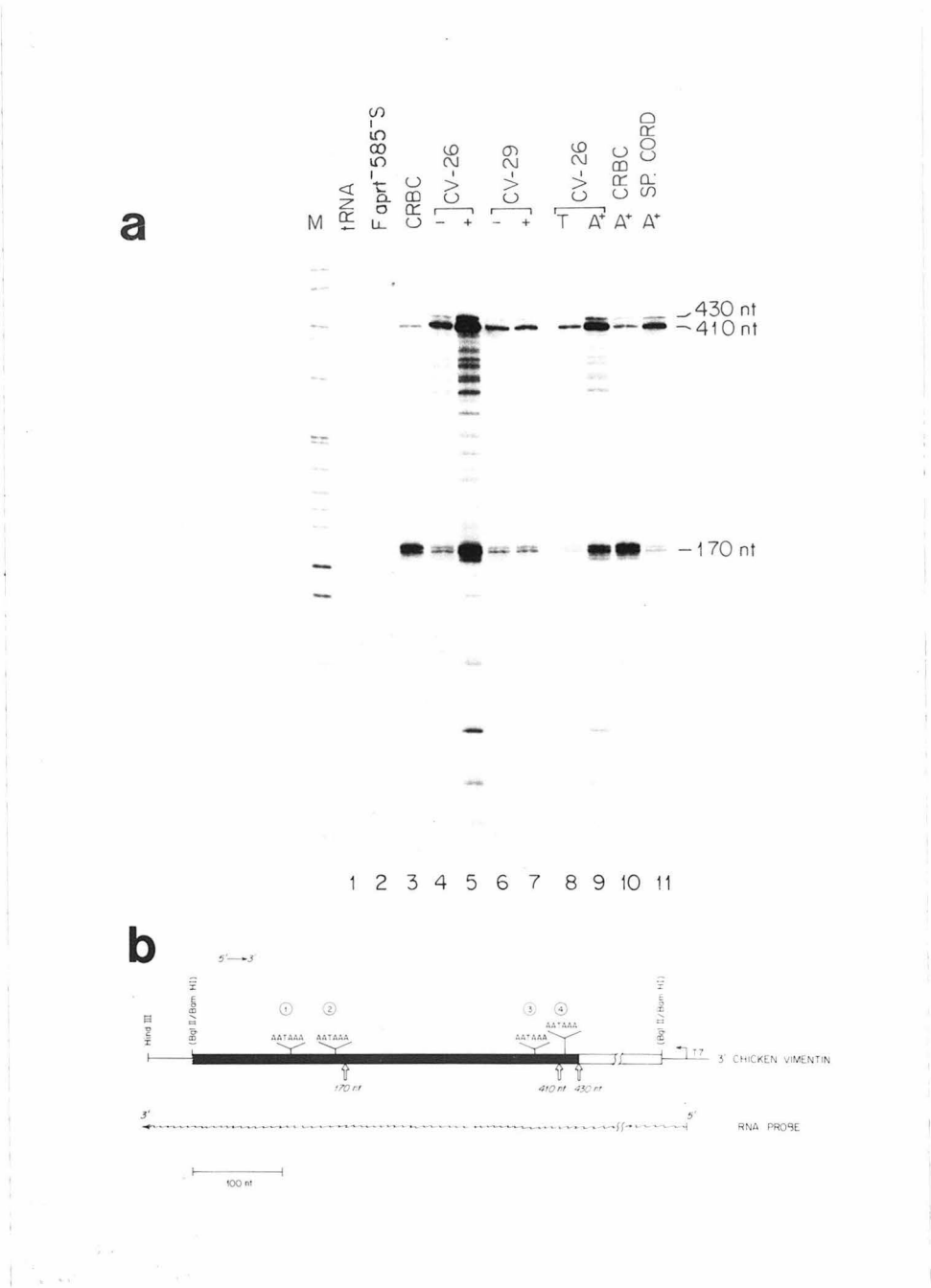
a
M
tRNA
F aprt⁻585⁻S
CRBC
CV-26
CV-29
CV-26
CRBC
SP. CORD
- + - + T A⁺ A⁺ A⁺
430 nt
410 nt
170 nt
1 2 3 4 5 6 7 8 9 10 11
b
5'→3'
Hind III
(Bgl II/Bam HI)
AATAAA
AATAAA
AATAAA
AATAAA
(Bgl II/Bam HI)
T7
3' CHICKEN VIMENTIN
170 nt
410 nt
430 nt
3'
5'
RNA PROBE
100 nt

Figure 4

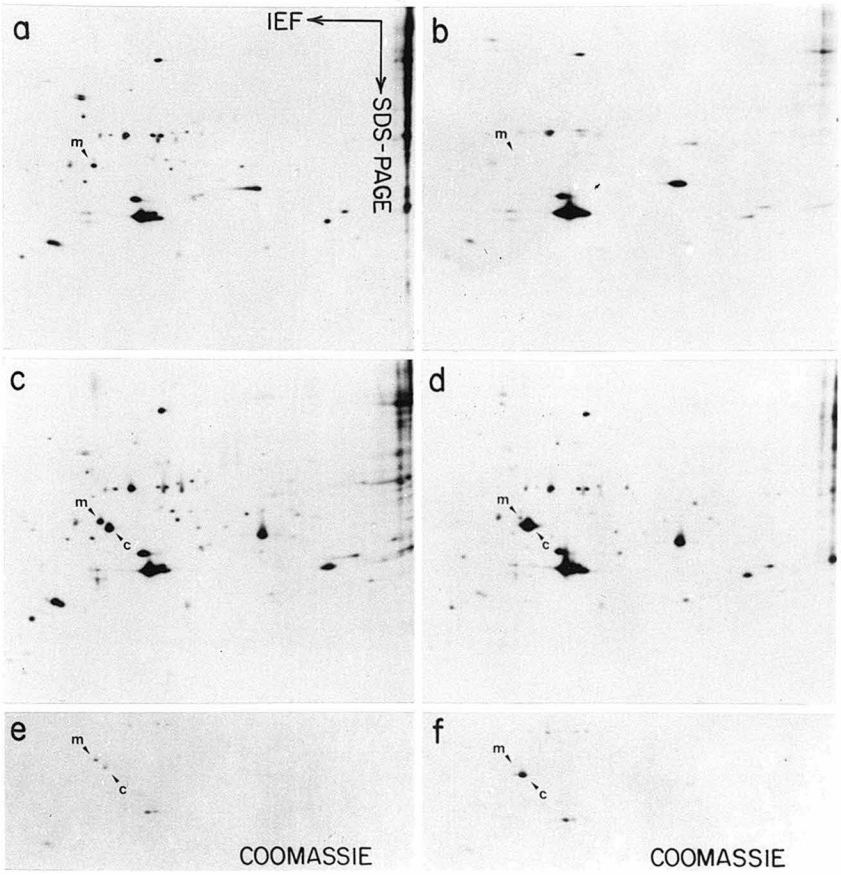

Figure 5

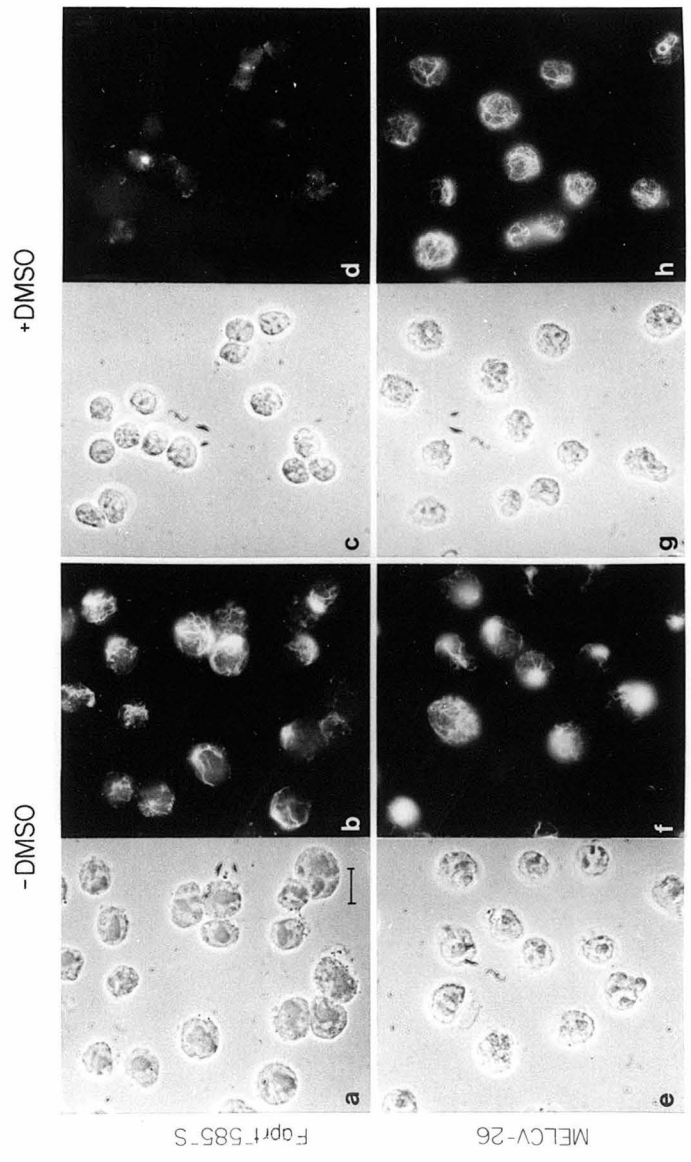

Figure 6

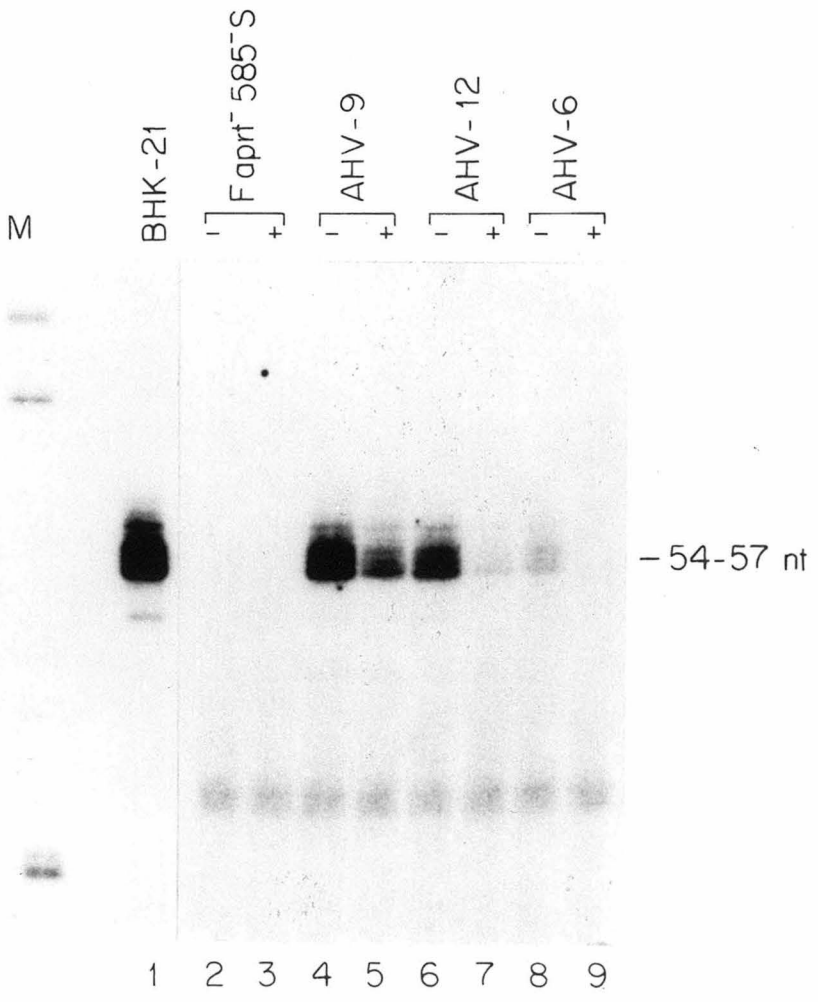

Figure 7

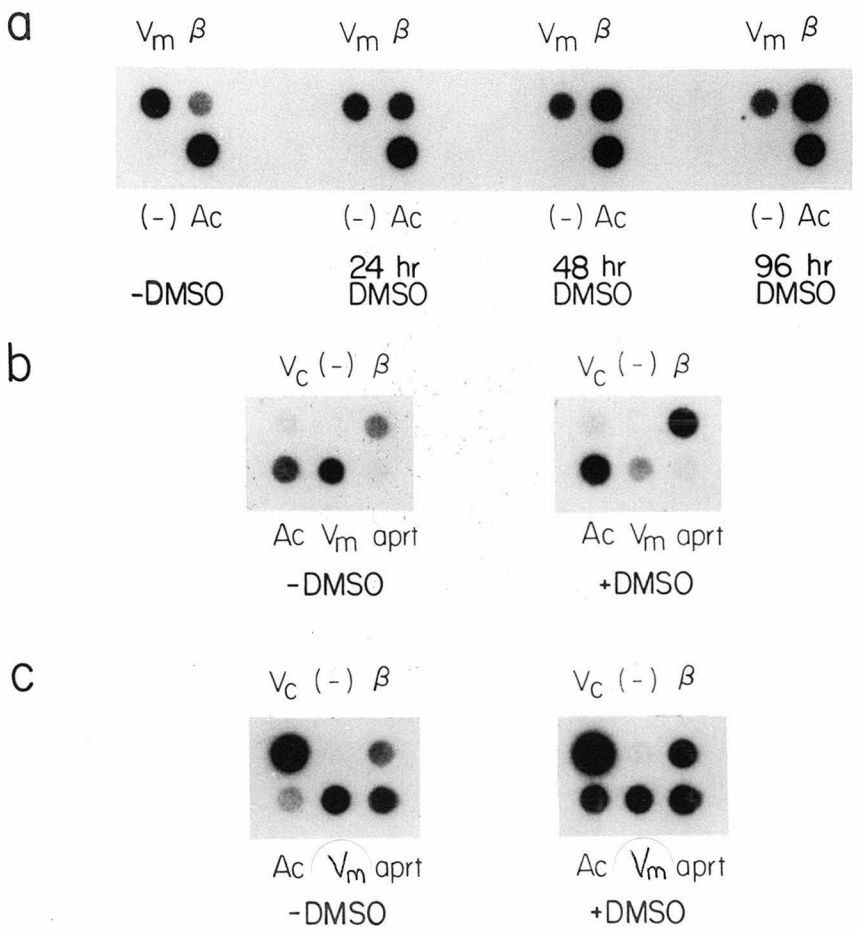
a
Vm β
Vm β
Vm β
Vm β
(-) Ac
(-) Ac
(-) Ac
(-) Ac
-DMSO
24 hr DMSO
48 hr DMSO
96 hr DMSO
b
Vc (-) β
Vc (-) β
Ac Vm aprt
Ac Vm aprt
-DMSO
+DMSO
c
Vc (-) β
Vc (-) β
Ac Vm aprt
Ac Vm aprt
-DMSO
+DMSO

Figure 8

CHAPTER 5:

Regulated Expression of Multiple Chicken Erythroid Membrane Skeletal Protein 4.1 Variants Is Governed by Differential RNA Processing and Translational Control

(In press, *Proceedings of the National Academy of Sciences, USA*, 1987)

Classification: Biochemistry

Regulated expression of multiple chicken erythroid membrane skeletal protein 4.1 variants is governed by differential RNA processing and translational control

(Key terms: erythropoiesis/membrane skeleton/multiple mRNAs)

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Communicated by Melvin I. Simon

ABSTRACT Protein 4.1 is an extrinsic membrane protein that facilitates the interaction of spectrin and actin in the erythroid membrane skeleton, and exists as a multiplet of structurally-related polypeptides in chickens. Previous studies have shown that the ratio of protein 4.1 variants is developmentally regulated during terminal differentiation of chicken erythroid and lenticular cells. To examine the mechanisms by which multiple chicken protein 4.1 variants are differentially expressed, we have isolated cDNA clones specific for chicken erythroid protein 4.1. We demonstrate that a single protein 4.1 gene gives rise to multiple 6.6 kilobase mRNAs by differential RNA processing. Furthermore, the ratios of protein 4.1 mRNAs change during chicken embryonic erythropoiesis. We observe a quantitative difference in variant ratios when protein 4.1 is synthesized *in vivo* or in a rabbit reticulocyte lysate *in vitro*. Together our results demonstrate that the expression of multiple protein 4.1 polypeptides is regulated both translationally and at the level of RNA processing.

INTRODUCTION

A network based on the proteins spectrin and actin lines the cytoplasmic surface of the mammalian erythrocyte plasma membrane, and is believed to confer upon this cell membrane its properties of strength and elasticity (1,2). Protein 4.1 facilitates the interaction of spectrin and actin by forming a ternary complex with these components, and also has been shown to interact directly with two intrinsic membrane proteins, glycophorin and the anion transporter, and membrane phospholipid (2). Protein 4.1 therefore plays a key role in the maintenance of the membrane skeleton. Protein 4.1 exists as a set of structurally related polypeptides in avian erythroid cells (3). In chicken erythrocytes, seven major protein 4.1 variants of 77, 87, 100, 115, 150, 160, and 175 kDa are expressed, with the 100 and 115 kDa polypeptides found in highest abundance (3,4). However, in immature mitotic erythroblasts, the 77 and 87 kDa protein 4.1 variants predominate, and as the cells undergo terminal differentiation, the 100 and 115 kDa forms are found at higher levels (4,5). Protein 4.1 and protein 4.1 analogues also have been localized in nonerythroid tissues (3,4,6-11).

In order to define the mechanism(s) by which multiple protein 4.1 variants are generated, we have isolated cDNA clones encoding chicken protein 4.1. Partial DNA sequence analysis demonstrates 86% homology with human protein 4.1 cDNA sequences (12) over a 270 bp portion of a chicken protein 4.1 cDNA. We find that the gene encoding protein 4.1 exists as a single copy in the haploid chicken genome, and gives rise to one 6.6 kb

size-class RNA. By S_1 nuclease mapping experiments, we demonstrate that this single size RNA consists of multiple, distinct mRNAs, whose expression is developmentally regulated during chicken embryonic erythropoiesis. Hence, a heterogeneity of protein 4.1 mRNAs appears to underlie the diversity of protein 4.1 polypeptides. A comparison of protein 4.1 polypeptides synthesized *in vitro* and *in vivo* demonstrates that the relative abundances of protein 4.1 variants is further regulated at the translational level.

MATERIALS AND METHODS

Isolation of protein 4.1 cDNAs. Protein 4.1-specific cDNAs were isolated from a λgt11 expression library constructed from 14-15 day old chick embryo erythroid poly(A)$^+$ RNA ("M" library of ref. 13). The library was screened with a protein 4.1-specific antiserum (3), and positive plaques were isolated and rescreened several times, as described (13). Complementary DNA inserts of candidate protein 4.1 clones were isolated after digestion of recombinant phage DNA with *Eco*RI and subcloned into pT7SP6 (a gift of Dr. V. Axelrod) (14) or m13mp 19. DNA sequence analysis was performed by the dideoxynucleotide chain termination procedure (15).

RNA preparation. Total cellular or cytoplasmic poly(A)$^+$ RNA was prepared from chicken embryonic erythroid cells as described previously (13). To enrich for protein 4.1 mRNA, cytoplasmic poly(A)$^+$ RNA was fractionated on a formamide-sucrose gradient, as described previously (13).

Fractions were collected, RNA was concentrated by ethanol precipitation, and fractions containing protein 4.1 translational activity in a rabbit reticulocyte lysate (16) were pooled.

***In vitro* translation and hybridization-selected translation.** Poly(A)$^+$ RNA was translated *in vitro* for 90 min at 30°C in a high activity nuclease-treated rabbit reticulocyte lysate containing ^{35}[S]methionine (13,16). Complete translation reactions or immunoprecipitates using a protein 4.1 antiserum (3) were resolved on 12.5% polyacrylamide NaDodSO$_4$ gels (17). For an *in vivo* protein 4.1 standard, 15-day old chick embryo erythroid cells were metabolically labeled with ^{35}[S]methionine for 30 min at 37°C, fractionated with Triton X-100, and immunoprecipitated with the protein 4.1 antiserum, as described (3). Labeled protein bands were visualized by fluorography of gels impregnated with 2,5-diphenyloxazole (18). Plasmid DNA was bound to nitrocellulose and subjected to positive hybridization-selected translation, as described (13,19). RNA selected from 10 μg of gradient enriched poly(A)$^+$ RNA was translated in 15 μl of rabbit reticulocyte lysate.

DNA and RNA analysis. DNA was digested with restriction endonucleases, electrophoresed, blotted to nitrocellulose, and protein 4.1 sequences were detected with ^{32}[P] nick translated cDNA inserts (20,21). RNA blots were performed as described previously (22,23). S_1 nuclease protection (24) of a protein 4.1 cDNA was performed by hybridizing RNA with an end-labeled 3.1 kb *Xho*I-*Pvu*I fragment of pFPO20, as described in the legend of Fig. 5.

RESULTS

Isolation of chicken protein 4.1 cDNAs. We identified cDNA clones coding for protein 4.1 from a λgt11 expression library (13) by screening with an antiserum specific for chicken protein 4.1 (3). DNA sequence analysis of the 5' 270 nt of one cDNA, pFPO20, reveals 86% nucleotide homology with a human reticulocyte protein 4.1 cDNA (12), rendering 98% amino acid sequence conservation from chicken to human protein 4.1 in this region (Fig. 1*a*). A restriction endonuclease map of representative protein 4.1 cDNAs is shown in Fig. 1*b*; we conclude from the above data that the cDNAs shown are specific for protein 4.1.

***In vitro* translation of chicken protein 4.1 and positive hybridization-selected translation.** To examine the mRNA(s) encoding the protein 4.1 polypeptides, we commenced by analyzing ^{35}[S]methionine-labeled translation products synthesized *in vitro* in a rabbit reticulocyte lysate (13,16), using cytoplasmic poly(A)$^+$ RNA isolated from circulating erythroid cells of 15-day old chick embryos. The seven major protein 4.1 variants of 77, 87, 100, 115, 150, 160, and 175 kDa all are immunoprecipitated from an *in vitro* translation reaction, with the 160 and 175 kDa polypeptides in greatest abundance (Fig. 2*a*, lane 2). However, when protein 4.1 is immunoprecipitated from cytoskeletal extracts of 15-day old embryo erythroid cells metabolically labeled with ^{35}[S]methionine, the 100 and 115 kDa polypeptides are the predominant forms, and the 77, 87, 150, 160, and 175 kDa variants are found in lower relative amounts (3-5) (see Fig. 2*a*,

lane 1 and Fig. 2*b*, lane 8). As the protein 4.1 polypeptides are rapidly stabilized in the membrane skeleton with equivalent efficiencies shortly after their synthesis (3-5), the pattern of protein 4.1 variants identified after labeling *in vivo*, shown in Fig. 2, closely approximates their ratios of synthesis, as well as the steady state ratios (3). The relative rates of synthesis of protein 4.1 variants *in vitro* and *in vivo* therefore differ significantly.

Protein 4.1 is a minor product of *in vitro* translations, and is not identifiable in the total translation reaction (compare Fig. 2*a*, lanes 2 and 3). To increase the relative abundance of protein 4.1 mRNA, we fractionated 15-day old embryo erythroid poly(A)$^+$ RNA on a denaturing 4-20% sucrose gradient, and pooled RNA fractions with protein 4.1 translational activity. Translation of this material *in vitro* shows that this preparation is substantially depleted of globin mRNA (Fig. 2*b*, lane 1; compare with Fig. 2*a*, lane 3), and directs the translation of the protein 4.1 polypeptides (Fig. 2*b*, lane 2). The 87, 100, and 115 kDa variants are more visible over a reduced background in the immunoprecipitation of the gradient-enriched RNA translation, as compared to translation of unfractionated RNA (Fig. 2*a*, lane 2). We therefore used gradient-enriched RNA for subsequent hybridization-selected translation experiments.

We performed hybridization-selected translations to further determine the structural relatedness of the protein 4.1 polypeptides. Fig. 2*b* demonstrates that a representative cDNA, pFPO2a, hybridizes to RNA which directs the synthesis of protein 4.1 polypeptides (lane 5). In contrast, the

plasmid vector alone does not hybridize to any detectable protein 4.1 RNA (lane 4). The hybridization-selected translation products of lane 5 are specifically immunoprecipitated by an anti-protein 4.1 antiserum (lane 7), whereas a similar immunoprecipitation of the plasmid vector-mediated selection is completely negative (lane 6). Positive hybridization-selected translation was repeated using pFPO2a, pFPO2b, and pFPO20, with results similar to those shown in Fig. 2 (data not shown). We conclude that the cDNAs described here, together representing ~3.5 kb, or 55% of protein 4.1 mRNA (see below), are complementary to the mRNA(s) encoding all the major protein 4.1 variants found in 15-day old embryo erythroid RNA.

Representation of protein 4.1 sequences in the chicken genome. Blot analysis of chicken genomic DNA using ^{32}P-labeled protein 4.1 cDNA probes demonstrates that the multiple protein 4.1 polypeptides are encoded by a single gene (Fig. 3). pFPO2a hybridizes to several genomic DNA bands generated by digestion with BamHI, HindIII, EcoRI, or XbaI (Fig. 3*a*). When the 5' 1.7 kb EcoRI-BamHI fragment or the 3' 0.7 kb BamHI-EcoRI fragment of pFPO2a is used as probe (see Fig. 1*a*), each hybridizes to a subset of the genomic bands obtained with the entire cDNA insert (Fig. 3*b*,*c*). A similar hybridization using pFPO2b as probe identifies the same BamHI-, HindIII-, and XbaI-genomic fragments detected by the pFPO2a 3' probe (Fig. 3*c*,*d*, lanes 1-3), but since pFPO2a and pFPO2b share a common terminal EcoRI site, pFPO2b hybridizes to a genomic EcoRI fragment which is not detected by pFPO2a (Fig. 3*a-d*, lanes 4). The hybridization patterns shown in Fig. 3*a-d*, in conjunction with a preliminary analysis of overlapping

protein 4.1 genomic DNA phage recombinants (unpublished observations) indicate that the multiple bands detected in protein 4.1 genomic DNA blots are contiguous; pFPO2a and pFPO2b hybridize to overlapping cloned genomic sequences spanning over 40 kb (unpublished observations). To determine the copy number of the protein 4.1 gene(s) in the chicken genome, known amounts of genomic DNA or DNA of a protein 4.1 genomic clone, λ4.1-19, were digested with EcoRI, and the relative hybridization of the 1.8 kb EcoRI fragment (an internal restriction fragment in the λ4.1-19 insert; data not shown) with ^{32}P-labeled pFPO2a was used to determine gene copy number (see Fig. 3, legend). The autoradiogram of Fig. 3*e* shows that the 1.8 kb EcoRI band detected (arrowhead) is found at one copy per haploid genome (compare lanes 1 and 6 or lanes 2 and 5). Since pFPO2a is complementary to RNA(s) encoding all the major protein 4.1 polypeptides (Fig. 2*b*, lanes 5 and 7), we conclude that multiple protein 4.1 variants arise from a single gene.

Multiple protein 4.1 mRNAs of indistinguishable sizes encode multiple chicken protein 4.1 polypeptides. The experiments shown in Figs. 2 and 3 together suggest that multiple protein 4.1 variants arise by translationally regulated expression of one or several mRNA(s) encoded by a single gene. RNA blot analysis of gradient-enriched 15-day old embryo erythroid poly(A)$^+$ RNA using ^{32}P-labeled pFPO2a as probe reveals a single major 6.6 kb RNA (Fig. 4*b*, lane I). Several minor bands smaller than the 6.6 kb RNA are also detected amidst a trail of degradation (Fig. 4*b*, lane I), in a pattern similar to that found in unfractionated poly(A)$^+$ RNA (data not shown). To determine if these minor species are protein 4.1 mRNAs or stable

degradation intermediates, we separated the gradient-enriched RNA on a preparative methylmercury hydroxide agarose gel, and analyzed gel-fractionated RNA by *in vitro* translation or by RNA blot analysis (Fig. 4). The autoradiograms of Fig. 4*a* and *b* demonstrate that translation of each major protein 4.1 variant co-fractionates in gel slices 4-7 (Fig. 4*a*), which corresponds to the major 6.6 kb protein 4.1 RNA peak (Fig. 4*b*). Within this peak, the ratios of the protein 4.1 polypeptides remain constant from fraction to fraction. The resolution of this experiment is sufficient to separate the streak of degraded protein 4.1 RNA, as well as the minor RNA species, from the protein 4.1 mRNA peak in slices 4-7. Hence, multiple protein 4.1 polypeptides are derived from a single size-class mRNA.

Although a single size mRNA appears to give rise to multiple protein 4.1 products, we could not rule out the existence of multiple protein 4.1 mRNAs with indistinguishable electrophoretic mobilities. We therefore performed S_1 nuclease digestions on hybrids formed between erythroid protein 4.1 RNA and an end-labeled protein 4.1 cDNA. For this purpose, a probe was prepared which consisted of a pFPO20 fragment end-labeled at the XhoI site (see Fig. 1*a*). Figure 5, lane 2 shows that the pFPO20 probe is protected by four distinct RNA species from erythroid cells of 15-day old chick embryos. The 1700 nt protected fragment identifies an RNA species complementary to pFPO20 from the XhoI site to the 3' terminus of the insert. Additional fragments are present at 730 nt, 580 nt, and 470 nt (barely visible in this exposure; see below), and represent RNAs which are derived from DNA containing this XhoI site, but are divergent 730,

580, and 470 nt downstream. S_1 nuclease analysis of RNA fractionated on the same methylmercury hydroxide agarose gel described in Fig. 4 demonstrates that the multiple protein 4.1 RNAs co-electrophorese at 6.6 kb (Fig. 4c), precluding the possibility that the multiplicity of protected fragments arises from RNA precursors or degradation products. A greater heterogeneity of protein 4.1 mRNA may well exist, as other putative RNAs which do not contain sequences both complementary to and continuous with the XhoI site within this 1.7 kb cDNA would not be detected.

Expression of multiple protein 4.1 mRNAs is developmentally regulated. Circulating erythroid cells of 4-day old embryos are "early" mitotic erythroblasts of the primitive series, whereas circulating red cells of 15-day old embryos are "late" post-mitotic definitive series cells (25). Previous studies have shown that the pattern of protein 4.1 variant expression in embryonic erythroid cells changes both during ontogeny as well as within the primitive series and definitive series lineages (4). To compare a "late" pattern of protein 4.1 mRNA expression (Fig. 5, lane 2) with a pattern from "early" cells, we performed S_1 nuclease analysis on RNA from 4-day old embryos. Using the ^{32}P-end-labeled probe described above, we find protection of only the 470 nt and 580 nt fragments by the 4-day old embryo erythroid RNA (Fig. 5, lane 3). Blot analysis of this RNA detects a single 6.6 kb protein 4.1-specific band (data not shown). We interpret the results described above to indicate that the generation of multiple protein 4.1 mRNAs is developmentally regulated.

DISCUSSION

We have examined the mechanisms by which multiple chicken protein 4.1 polypeptides are generated, using cloned cDNA probes specific for protein 4.1. Our protein 4.1 cDNAs are complementary to RNAs encoding all the major chicken erythroid protein 4.1 polypeptides, as shown by hybridization-selected translations. Quantitative genomic DNA blotting reveals that multiple protein 4.1 variants arise from a single gene. By RNA blot analysis and S_1 nuclease mapping, we have determined that the single protein 4.1 gene generates multiple mRNAs with indistinguishable sizes. The observation of multiple 6.6 kb mRNAs exhibiting sequence discontinuities at distances of up to ~1 kb apart indicates that alternative pathways of pre-mRNA splicing (e.g., see refs. 26-28) play a key, but not necessarily exclusive role in the genesis of multiple protein 4.1 mRNAs. Different sites of transcription initiation, as well as termination and/or transcript cleavage and polyadenylation may also occur, and indeed may determine the selection of splicing patterns, as shown for other transcription units (e.g., see refs. 29-31).

In this study we demonstrate that the differential expression of multiple protein 4.1 mRNAs is developmentally regulated, as the mRNAs detected switch from "early" primitive cells to "late" definitive cells. This switch is paralleled by changes in protein 4.1 polypeptide synthesis and accumulation (4). Translational and/or co-translational processes also regulate the pattern of protein 4.1 expression, as demonstrated by the quantitative difference in protein 4.1 polypeptide synthesis *in vivo* and

in vitro. The relative amounts of protein 4.1 variants may be controlled by preferential translation of certain protein 4.1 mRNAs. Alternatively, limited co-translational or rapid post-translational processing of the larger protein 4.1 polypeptides may give rise to the smaller variants (see refs. 3,5). Hence, although expression of protein 4.1 polypeptides is specified initially at the mRNA level by RNA processing, the relative abundance, or perhaps even the presence or absence of each variant is further determined by translational or co-translational mechanisms.

Our data regarding the expression of multiple protein 4.1 mRNAs from a single gene may be applicable to the observed occurrence of protein 4.1 and protein 4.1 analogues in non-erythroid tissues (3,4,6-9,11). For example, in developing chick lens, protein 4.1 variants similar to those found in chicken erythroid cells are present (3,4). A 6.6 kb protein 4.1 mRNA is detected in chick lens by RNA blotting (data not shown), suggesting that lens protein 4.1 is specified by the same gene that encodes erythroid protein 4.1. The significance of producing multiple protein 4.1 mRNAs and polypeptides in a developmentally-regulated and tissue-specific manner remains to be determined. It is possible that differential splicing of protein 4.1 exons facilitates the expression of alternative functional domains, which would affect or mediate the interactions of protein 4.1 with other membrane skeletal components. Future studies employing full-length protein 4.1 cDNAs should facilitate our understanding of both the structural and regulatory features of the differential expression of multiple protein 4.1 mRNAs and polypeptides.

Mammalian erythroid protein 4.1 exists as two structurally related polypeptides with molecular masses of 80 kDa (4.1a) and 78 kDa (4.1b) (32,33); protein 4.1a and 4.1b differ at their carboxy-termini, but appear to be functionally equivalent (33). Mammalian lens generally expresses a minor 125-145 kDa protein 4.1-related polypeptide in addition to the protein 4.1a/b doublet (3,6,11) Hence, mammalian protein 4.1 is expressed as a number of related variants, but the extent of heterogeneity is lesser, or perhaps more subtle than that found in avian species.

We thank Drs. Bruce Granger, Barbara Wold and John Cox for helpful discussions during the course of this study. This work was supported by grants from the NIH, NSF, and Muscular Dystrophy Association of America. J.N. was also supported by a Gordon Ross Foundation Fellowship, J.H.S by a training grant from the National Institutes of Health (T32 GM07616), and R.T.M. by an American Cancer Society postdoctoral fellowship.

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Figure Legends

FIG. 1 Protein 4.1 cDNAs. *a)* DNA sequence analysis of the 5' 270 nt of pFPO20. Top row: sequence of a human protein 4.1 cDNA from nucleotide 1324 to 1593, according to Conboy *et al.* (12). Middle row: sequence of pFPO20, determined by Jeffrey Stack. Dots indicate non-homologous nucleotides. Bottom row: Amino acid sequence derived from the chicken protein 4.1 cDNA. Non-homologous human protein 4.1 residues are indicated in parentheses. *b)* Restriction endonuclease map of protein 4.1 cDNAs. pFPO2a and pFPO2b were isolated from the same λgt11 recombinant (λFPO-2). EcoRI sites in parentheses are from synthetic linkers, whereas the EcoRI site shared by pFPO2a and pFPO2b occurs naturally. The direction of transcription of pFPO2a and pFPO2b was determined by their orientation within the β-galactosidase gene of λgt11 (34). pFPO20 was isolated subsequent to back-screening of the λ cDNA library with the pFPO2a insert.

FIG. 2. Synthesis of protein 4.1 *in vivo* and *in vitro* and identification of protein 4.1-specific cDNAs. *a)* $NaDodSO_4$ polyacrylamide gel electrophoretic analysis of protein 4.1 synthesis. Lane 1, immunoprecipitate of protein 4.1 from cytoskeletal extracts of 15-day old embryo erythroid cells labeled *in vivo*. Lane 2, immunoprecipitation from 10 μl rabbit reticulocyte lysate in which of 15-day old embryo erythroid cytoplasmic poly(A)$^+$ RNA was translated. Lane 3, one microliter of the complete *in vitro* translation reaction used in lane 2. The high molecular weight triplet in lane 3 is

ankyrin, α-spectrin, and β-spectrin, in descending order. Bars indicate positions of protein 4.1 variants (from top to bottom): 175 kDa, 160 kDa, 150 kDa, 115 kDa, 100 kDa, 87 kDa, and 77 kDa. Asterisk marks the position of the 70 kDa heat shock protein, which is a major translation product of 15-day-old embryo red cell RNA, and is immunoprecipitated non-specifically from the *in vitro* translation reaction. Lanes 1 and 2 were fluorographed for 8 days, whereas lane 3 was exposed for 16 hr. *b*) $NaDodSO_4$-polyacrylamide gel analysis of gradient-enriched erythroid poly(A)$^+$ RNA translation and hybridization-selected translation. Lane 1, 0.5 μl of an *in vitro* translation of gradient-enriched 15-day-old embryo erythroid poly(A)$^+$ RNA. Lane 2, immunoprecipitation from 10 μl of the *in vitro* translation reaction shown in lane 1. Lane 3, endogenous translational activity from 5 μl lysate with no added RNA. Lane 4, hybridization-selected translation using plasmid vector (5 μl of a 15 μl reaction). Lane 5, hybridization-selected translation mediated by pFPO2a (5 μl). Lanes 6,7, protein 4.1-specific immunoprecipitation from 10 μl lysate of lanes 4 and 5, respectively. Lane 8: Immunoprecipitate of metabolically-labeled protein 4.1, as in *a*, lane 1. Bars indicate positions of protein 4.1 polypeptides, as in *a*. Lanes 1-8 represent consecutive lanes from the same gel, but were fluorographed for 1 day (lane 1), 3 days (lanes 2-5), and 6 days (lanes 6-8).

FIG. 3. Blot analysis of chicken genomic DNA using protein 4.1 cDNA probes. *a–d*) One microgram of chicken liver DNA was digested with BamHI (lane 1), HindIII (lane 2), EcoRI (lane 3), or XbaI (lane 4) electrophoresed on a

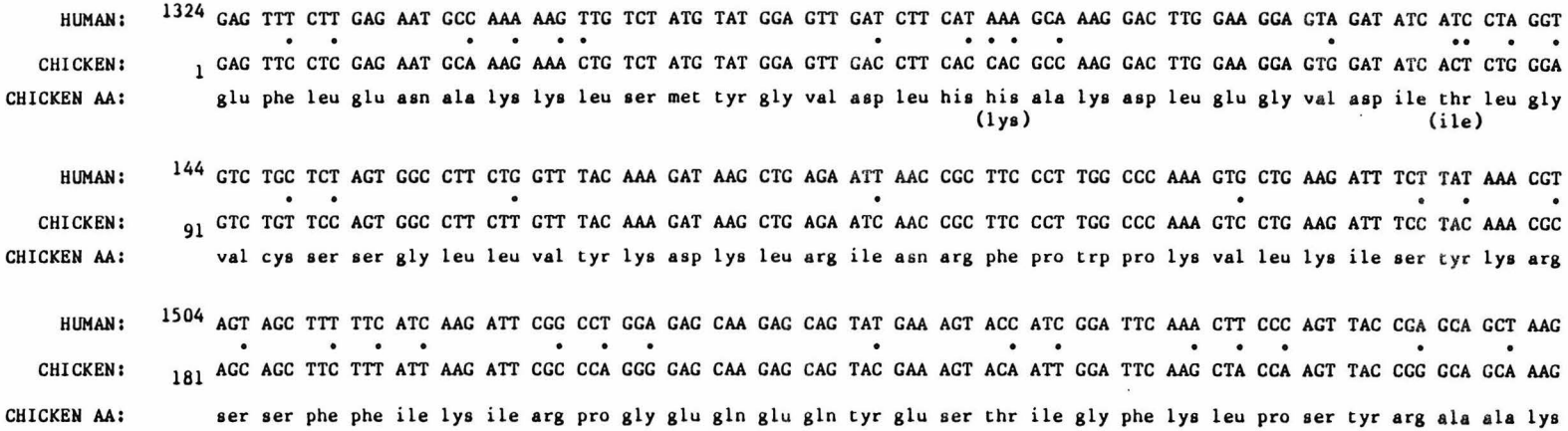
0.9% agarose gel, and blotted to nitrocellulose. Blots were hybridized to the following ^{32}P-labeled nick-translated cDNA sequences: *a*) pFPO2a insert, *b*) 1.7 kb pFPO2a 5' EcoRI-BamHI fragment, *c*) 0.7 kb pFPO2a 3' BamHI-EcoRI fragment, and *d*) pFPO2b insert. The autoradiograms in panels *a-d* were from adjacent strips of nitrocellulose blotted to the same gel. The positions of λ DNA digested with HindIII is shown (23, 9.4, 6.6, 2.3, 2.0, and 0.56 kb). *e*) Determination of protein 4.1 gene copy number. A recombinant phage, λ4.1-19, containing chicken protein 4.1 gene sequences was isolated from a λ Charon 4A genomic library (35) and used to quantitate protein 4.1 DNA hybridization. Chicken liver DNA (1.0 μg and 0.5 μg) and λ 4.1-19 DNA were digested with EcoRI, separated on a 0.9% agarose gel, blotted to nitrocellulose, and hybridized to ^{32}P-labeled pFPO2a. The 1.8 kb EcoRI band (arrowhead) was used to calibrate the hybridization signals. Lane 1, 10 μg chicken liver DNA. Lane 2, 0.5 μg chicken liver DNA. Lanes 3-8, λ4.1-19 DNA corresponding to 0.25, 0.5, 1.0, 2.0, and 4.0 copies per haploid genome (C = 1.5 pg) per μg chicken DNA, respectively. Bars denote λ *Hin*dIII DNA size markers.

FIG. 4. Fractionation of 15-day-old embryo erythroid RNA on a methylmercury hydroxide agarose gel and analysis by *in vitro* translation, RNA blotting, and S_1 nuclease protection. Gradient-enriched poly(A)$^+$ RNA was electrophoresed in the presence of methylmercury hydroxide (36) on a preparative 0.9% low gelling temperature agarose gel. The gel was sliced in 0.5 mm thick sections perpendicular to the axis of electrophoresis, and RNA

was isolated as described previously (22). *a*) *In vitro* translation of fractionated RNA. RNA was translated in a rabbit reticulocyte lysate containing ^{35}[S]methionine, immunoprecipitated with protein 4.1 antiserum, and analyzed on a 12.5% polyacrylamide-NaDodSO$_4$gel. C, immunoprecipitate of protein 4.1 from 15-day-old embryo erythroid cells labeled with ^{35}[S]methionine. I, immunoprecipitation from reticulocyte lysate in which gradient-enriched poly(A)$^+$ RNA ("input") was translated. Lanes 1-15, immunoprecipitates from translations of fractionated RNA from gel slices 1-15, respectively. Bars indicate 175, 150, 115, 100, and 87 kDa protein 4.1 variants. *b*) RNA blot of fractionated RNA. RNA was electrophoresed in the presence of formaldehyde, blotted to nitrocellulose, and probed with ^{32}P-labeled pFPO2a cDNA insert. I, gradient-enriched RNA ("input"). Lanes 1-15, RNA from slices 1-15. *c*) S_1 nuclease analysis of fractionated RNA. S_1-nuclease mapping was performed using a ^{32}P-end-labeled pFPO20 cDNA probe (see Fig. 5 legend). Protected fragments were separated on a 1.5 mm-thick 7 M urea-5% polyacrylamide gel. tRNA, ^{32}P-labeled DNA protected after incubation with tRNA. I, fragments protected by gradient-enriched poly(A)$^+$ RNA ("input"). Lanes 2-9, fragments protected by RNA in slices 2-9.

FIG. 5. S_1 nuclease protection of protein 4.1 cDNA by chick erythroid RNA. pFPO20 was cleaved with XhoI and end-labeled by filling out with DNA polymerase I large fragment in the presence of α-^{32}[P]dNTPs (see Fig. 1*b*), digested with PvuI, and the 3.1 kb fragment containing 1.7 kb insert

plus 1.4 kb vector sequences was isolated and hybridized with RNA in 80% formamide, 40 mM PIPES, 0.4 M NaCl, 1 mM EDTA at 50°C for 15 hr. Samples were digested with S_1 nuclease (24), and fragments were resolved on 0.3 mm-thick denaturing 7 M urea-5% polyacrylamide gels. Lane 1, undigested probe. Lane 2, fragments protected by 2 μg cytoplasmic poly(A)$^+$ RNA of 15-day-old embryo erythroid cells. Lane 3, fragments protected by 2 μg total cellular poly(A)$^+$ RNA of 4-day old embryo erythroid cells. Lane 4, S_1 nuclease digestion following hybridization with 2 μg tRNA. Bars indicate mobilities of end-labeled DNA markers (1856, 1060, 929, 622, 527, 404, 383, 309, 242, 238, and 217 nt).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CHICKEN: 1 GAG TTC CTC GAG AAT GCA AAG AAA CTG TCT ATG TAT GGA GTT GAC CTT CAC CAC GCC AAG GAC TTG GAA GGA GTG GAT ATC ACT CTG GGA
CHICKEN AA: glu phe leu glu asn ala lys lys leu ser met tyr gly val asp leu his his ala lys asp leu glu gly val asp ile thr leu gly
(lys) (ile)
HUMAN: 144 GTC TGC TCT AGT GGC CTT CTG GTT TAC AAA GAT AAG CTG AGA ATT AAC CGC TTC CCT TGG CCC AAA GTG CTG AAG ATT TCT TAT AAA CGT
CHICKEN: 91 GTC TGT TCC AGT GGC CTT CTT GTT TAC AAA GAT AAG CTG AGA ATC AAC CGC TTC CCT TGG CCC AAA GTC CTG AAG ATT TCC TAC AAA CGC
CHICKEN AA: val cys ser ser gly leu leu val tyr lys asp lys leu arg ile asn arg phe pro trp pro lys val leu lys ile ser tyr lys arg
HUMAN: 1504 AGT AGC TTT TTC ATC AAG ATT CGG CCT GGA GAC CAA GAG CAG TAT GAA AGT ACC ATC GGA TTC AAA CTT CCC AGT TAC CGA GCA GCT AAG
CHICKEN: 181 AGC AGC TTC TTT ATT AAG ATT CGC CCA GGG GAG CAA GAG CAG TAC GAA AGT ACA ATT GGA TTC AAG CTA CCA AGT TAC CGG GCA GCA AAG
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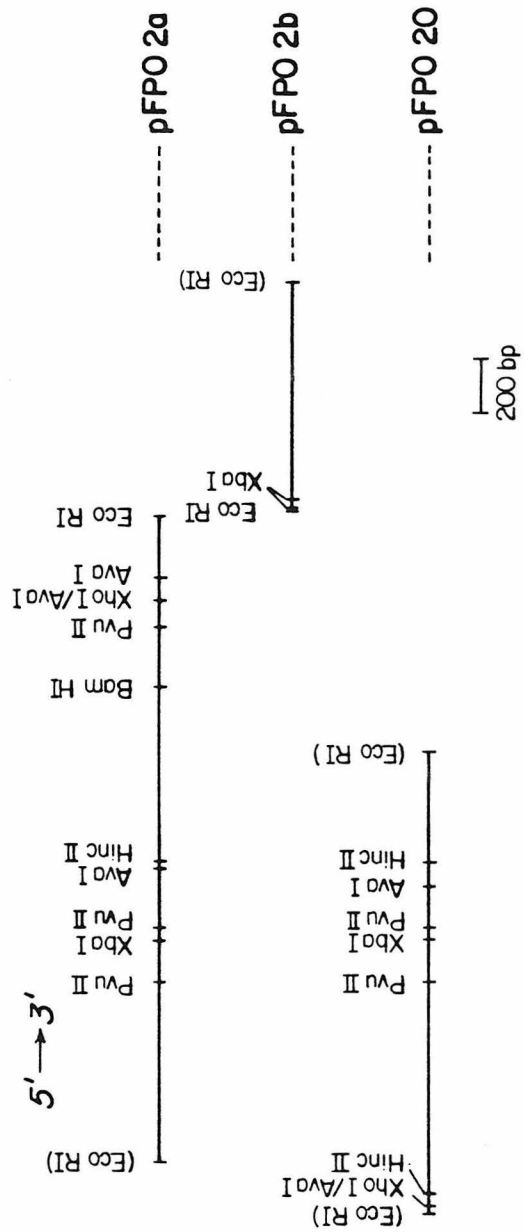
Figure 1a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pFPO 2b
pFPO 20
(Eco RI)
200 bp
Xba I
Eco RI
Eco RI
Bam HI
Pvu II
Xho I/Avo I
Avo I
Hinc II
Avo I
Xba I
Pvu II
Pvu II
5' → 3'
(Eco RI)
(Eco RI)
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Avo I
Pvu II
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Xho I/Avo I
Hinc II
(Eco RI)

Figure 1b

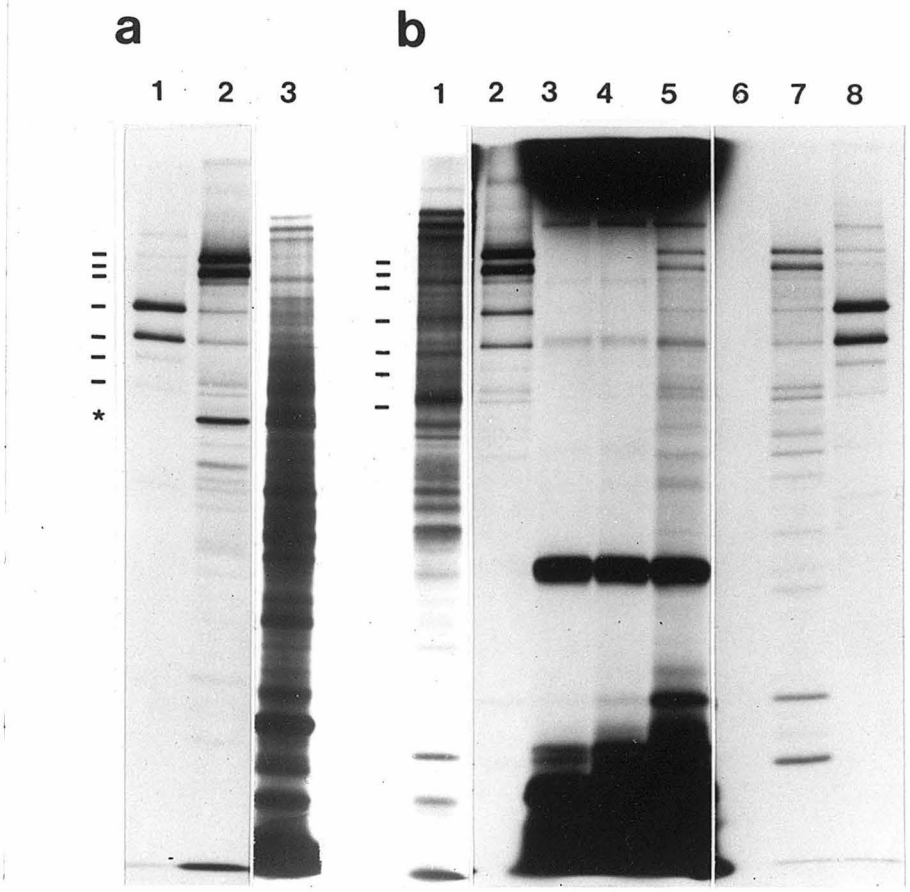
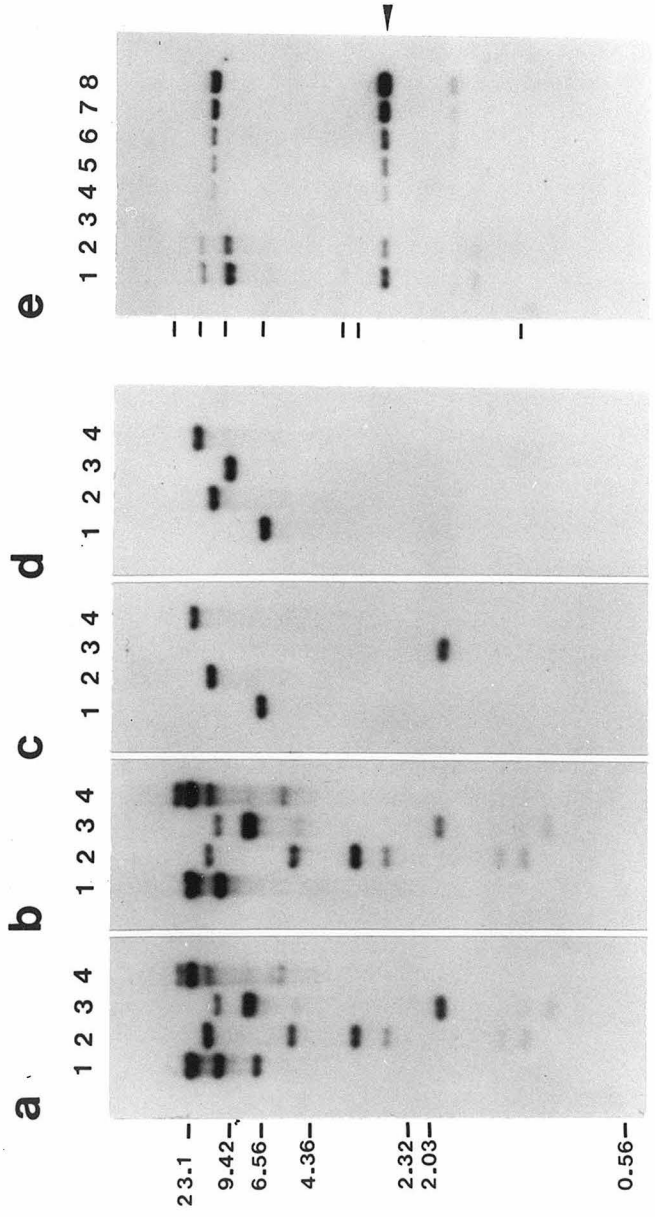


Figure 2


a
b
c
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e
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1 2 3 4
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23.1
9.42
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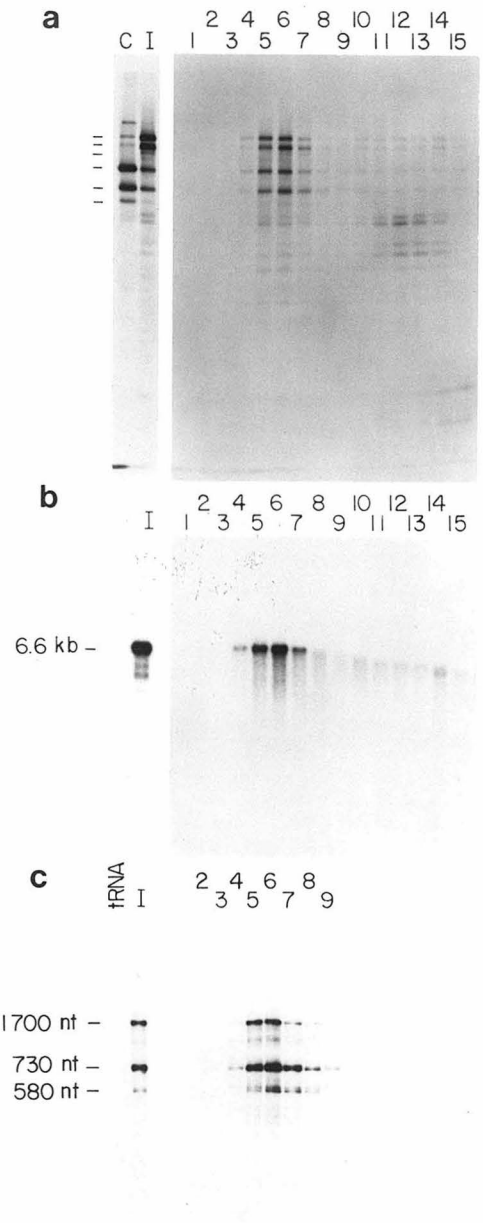
2.32
2.03
0.56

Figure 3


a
C I
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b
I
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
6.6 kb –
c
tRNA
I
2 3 4 5 6 7 8 9
1700 nt –
730 nt –
580 nt –

Figure 4

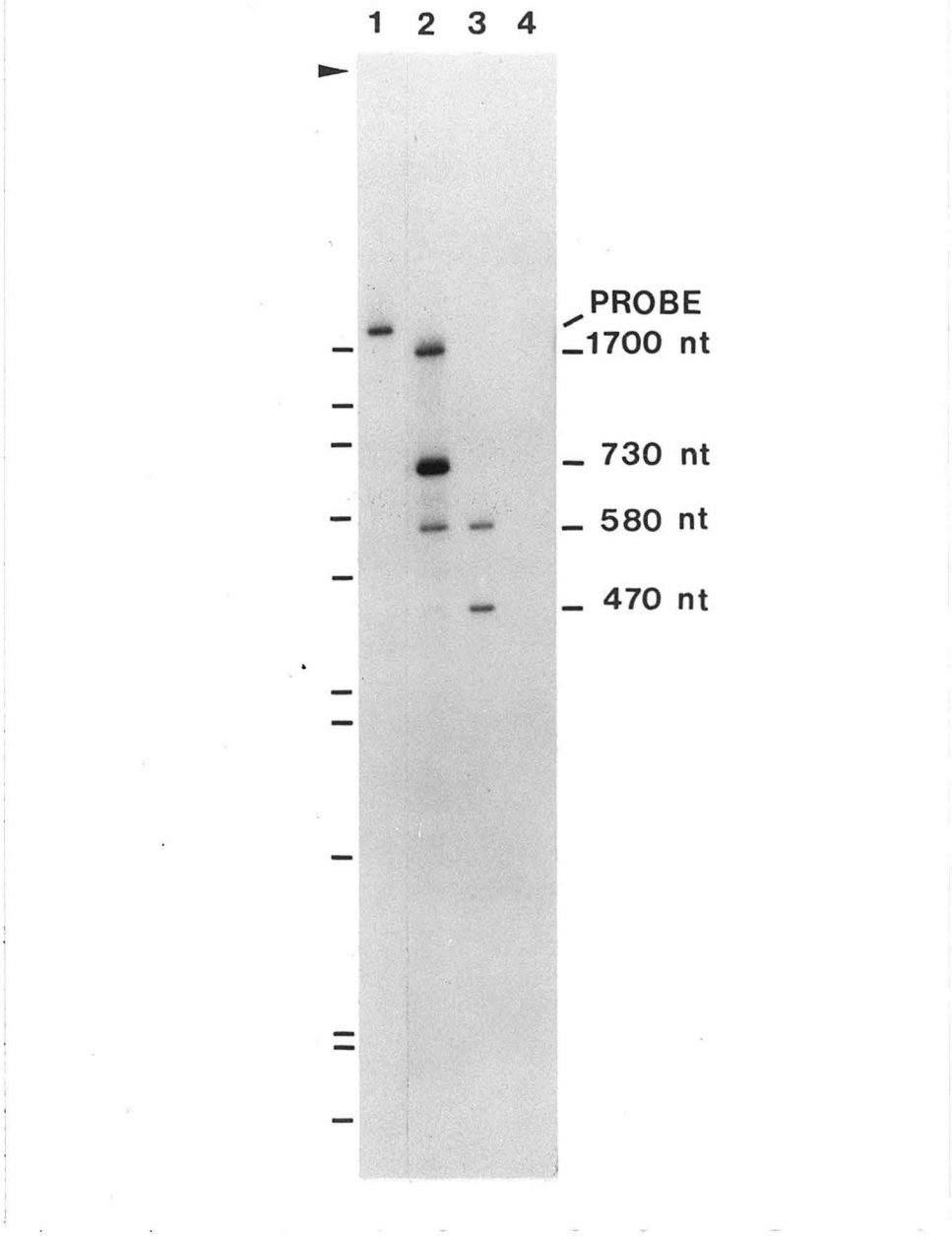

Figure 5

CHAPTER 6:

Conclusions

The study of vimentin expression during cellular differentiation has revealed several interesting aspects of the regulation of the gene encoding this intermediate filament protein. The chicken vimentin gene produces three distinct mRNAs which differ in the lengths of their 3' untranslated regions (Zehner and Paterson, 1983a,b). Size heterogeneity of 3' untranslated sequences has also been demonstrated for a number of other mRNAs, for example, mouse dihydrofolate reductase (Setzer et al., 1980), mouse β_2-microglobulin (Parnes and Robinson, 1983), mouse α-amylase (Tosi et al., 1981), and eel calmodulin (Legacé et al., 1983) mRNAs. Although the effects of variations in the lengths of chicken vimentin RNA 3' untranslated regions is not known, the cell- and tissue-specific utilization of chicken vimentin polyadenylation sites (Capetanaki et al., 1983 [Chapter 2]) implies that the resulting differences are functionally significant. The differential utilization of polyadenylation sites may affect the relative stabilities or posttranscriptional processing of chicken vimentin mRNAs. It is also possible that chicken vimentin 3' untranslated sequences may affect subcellular compartmentation differentially. Lawrence and Singer (1986) have shown by in situ hybridization that vimentin RNA exhibits a preferential nuclear or peri-nuclear localization in primary chick embryo fibroblasts. However, in these experiments vimentin RNA was detected using a probe complementary to all three mRNAs, and so discrimination of the localization of any specific mRNA species was not possible. The contribution of chicken vimentin 3' untranslated sequences to subcellular mRNA localization remains an interesting possibility to be explored. It

should again be noted that mammalian cells express only one vimentin mRNA species; a comparison of hamster and chicken vimentin mRNAs demonstrates 83% nucleotide sequence homology in the 3' untranslated regions, with the single hamster polyadenylation signal corresponding to the second chicken vimentin signal (Quax et al., 1983). Any explanation for the importance of cell-specific expression of multiple vimentin mRNAs with different 3' untranslated regions therefore must be reconciled with the presence of only one vimentin mRNA in mammals. However, it is possible that a functional requirement for multiple vimentin mRNAs in mammals has been lost or altered since the divergence of birds and mammals, as suggested by the differences in posttranscriptional regulation of mouse and chicken vimentin mRNAs in differentiating MEL cells (Chapter 4).

The developmental and tissue-specific expression of vimentin is complex. During terminal differentiation, vimentin may be completely repressed, as in chick spinal cord neurons (Tapscott et al., 1983a,b), mammalian erythropoiesis (Dellagi et al., 1983; Ngai et al., 1984 [Chapter 3]), and mammalian B lymphocyte development (Dellagi et al., 1983; McTavish et al., 1983; Traub et al., 1983); partially repressed or constitutively expressed, as in myogenesis (Granger and Lazarides, 1979; Gard and Lazarides, 1980), astrocytes (Tapscott et al., 1981b; Yen and Fields, 1981; Schnitzer et al., 1981), and certain retinal neurons (Dräger, 1983); or induced, as in avian erythropoiesis (Capetanaki et al., 1983 [Chapter 2]). How is vimentin differentially regulated in such a diverse variety of cells? Perhaps there are multiple tissue-specific *cis*-linked

regulatory sequences that are responsible for the positive or negative regulation in these differentiating cell lineages. For example, multiple tissue-specific regulatory elements have been demonstrated to be responsible for the expression of the mouse α-fetoprotein gene in yolk sac visceral ectoderm, fetal liver, or gut (Godbout et al., 1986; Hammer et al., 1987). A preliminary indication of a multiplicity of vimentin regulatory sequences is implied by the differences in both transcriptional and post-transcriptional regulation of chicken and mouse vimentin RNA levels in differentiating MEL cells (Chapter 4). The divergence of mammalian and avian vimentin gene expression in erythropoiesis should continue to be useful in delineating the *cis*-linked regulatory sequences responsible for the developmental regulation of this gene. For example, the expression of hybrid hamster-chicken vimentin fusion genes in differentiating MEL cells may help to identify such putative positively- and negatively-responding sequences.

In Chapter 5 I have presented evidence for differential RNA processing and translational regulation as mechanisms governing the expression of multiple chicken membrane skeletal protein 4.1 polypeptides. Although the functional significance of multiple protein 4.1 variants is at present unknown, the future isolation and characterization of full-length protein 4.1 cDNAs will aid in the elucidation of protein 4.1 isoform structure.

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CHAPTER 7:

Appendix: Expression of the Genes Coding for the Intermediate Filament Proteins Vimentin and Desmin

(Appeared in *Annals of the New York Academy of Sciences*, Vol., 455, pp. 144-157, 1985)

Expression of the Genes Coding for the Intermediate Filament Proteins Vimentin and Desmin[a]

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INTRODUCTION

Patterns of intermediate filament protein expression have provided several model systems in which to study the regulation of both gene expression and morphogenesis during cell differentiation. In a variety of cell types, a specific intermediate filament subunit is induced as part of a terminal differentiation program. For example, vimentin is expressed as the major intermediate filament protein in the avian erythrocyte[1] and lens fiber cell.[2,3] In other cases, vimentin is present during immature stages and precedes the appearance of the cell type-specific subunit. During terminal differentiation, the particular subunit may partially or completely replace vimentin as the major intermediate filament protein. In developing chick spinal cord, neurofilament protein is expressed in lieu of vimentin during the terminal differentiation of neurons.[4,5] In chicken myogenesis, vimentin is coexpressed with desmin, whose synthesis is induced following fusion of myoblasts in the formation of multinucleate myotubes;[6,7] the coexpression of vimentin and desmin persists in adult chicken skeletal muscle.[8] Heterogeneity of intermediate filament composition within a cell type is also seen in certain mouse retinal neurons, where vimentin coexists with neurofilament protein,[9] and in astrocytes, where glial fibrillary acidic protein (GFAP) and vimentin are both present.[5,10,11] Interestingly, the 70,000 dalton core neurofilament polypeptide (NF70) has been found in sub-stoichiometric quantities relative to vimentin in erythrocytes from chicken embryos and young chicks.[12] Intermediate filament proteins therefore represent a class of proteins whose expression is differentially regulated both within and between specific cell lineages. Furthermore, the changing composition of intermediate filaments

[a] This work was supported by grants from the National Institutes of Health, National Science Foundation, Muscular Dystrophy Association of America and a Grant-in-Aid from the American Heart Association, Greater Los Angeles Affiliate. J.N. was also supported by a Gordon Ross Foundation Predoctoral Fellowship, Y.G.C. by Muscular Dystrophy Association and Proctor and Gamble postdoctoral fellowships. E.L. is the recipient of a Research Career Development Award from the National Institutes of Health.

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in some differentiating cells suggests changing functional requirements for these filaments.

To examine the regulation of intermediate filament proteins and their genes, we have begun by studying vimentin and desmin expression during avian and mammalian erythropoiesis and during myogenesis *in vitro*. Recently we have approached these systems at the molecular level using recombinant DNA clones for chicken vimentin and desmin.[13–15] In this paper we will review the results from these studies and will attempt to place them in the broader context of intermediate filament expression to help define their modes of regulation.

Expression of the Chicken Vimentin Gene

We have shown that two mRNA size classes arise from the single vimentin gene[16] with cell- and tissue-specific modes of expression.[13] Vimentin mRNAs with lengths of 2.0 and 2.3 kilobases (kb) are both present in chicken skeletal muscle, lens, spinal cord, cultured embryo fibroblasts and myogenic cells, and at low levels in gizzard and 4-day embryonic erythroid cells.[13] The ratios of the two mRNA classes vary, however; for example, there are roughly equal amounts of 2.0 and 2.3 kb vimentin mRNAs in skeletal muscle, whereas in spinal cord, the 2.3 kb RNA is 1.5-2 times

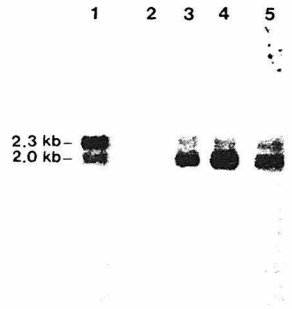

FIGURE 1. Tissue-specific expression of the two chicken vimentin mRNAs. Polyadenylated RNA was separated by electrophoresis in the presence of formaldehyde, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled vimentin probe.[13] Lane 1, 3 μg spinal cord RNA; lane 2, 3 μg 4-day embryonic erythroid RNA; lane 3, 3 μg 10-day embryonic erythroid RNA; lane 4, 3 μg 15-day embryonic erythroid RNA; lane 5, same as lane 2, but exposed for a sevenfold longer period of time. Note differences in the relative ratios of the 2.3 kb and 2.0 kb RNAs (see text).

as prevalent, and in 4-day embryonic erythrocytes, the 2.0 kb RNA is approximately twice as abundant as the 2.3 kb RNA. In contrast to the abovementioned cases, erythroid cells from 10- and 15-day embryos express predominantly the 2.0 kb mRNA species[13] (FIGURE 1). Furthermore, there is a ~20-fold greater accumulation of vimentin mRNA in erythrocytes from 10-day-old embryos as compared to erythrocytes from 4-day-old embryos, and a further ~twofold increase from 10-day to 15-day embryonic erythroid cells (FIGURE 1).

Variations in the lengths of 3′ untranslated regions appear to be primarily responsible for the ~300 nucleotide (nt) difference between the two chicken vimentin mRNA classes.[13,16,17] Sequence data of the 3′ end of the gene have revealed two pairs of putative polyadenylation sites (sequence: 5′-AATAAA-3′) within the 3′ untranslated region separated by ~250 nt.[16] These findings suggest that different 3′ termini of vimentin mRNAs account for all or most of the length discrepancies observed between

the two mRNA classes. We have shown directly by hybridizing different restriction fragments of the cloned vimentin gene to RNA blots that the 2.0 and 2.3 kb RNAs indeed differ at their 3′ ends.[13] Hence, a probe generated from the 3′ terminal region of the vimentin gene hybridizes only to the 2.3 kb mRNA, whereas sequences upstream hybridize to both RNA classes (FIGURE 2). Preliminary S_1 nuclease mapping at the 5′ end of the gene has revealed no differences between the two RNAs at our level of resolution (~50 bp) (Capetanaki, Ngai, and Lazarides, unpublished results). S_1 nuclease mapping experiments by Zehner and Paterson have confirmed the utilization of three of the four possible poly(A) addition sites in chicken embryonic muscle vimentin mRNA.[17] From the available sequence data[16] and the experiments described above,[13,17] the usage of differential splicing patterns to generate multiple 3′ terminal sequences is unlikely. Moreover, it appears that the vimentin mRNA species share common protein-coding sequences, and the differences observed reside in the lengths of 3′ untranslated regions. These differences may be generated either by transcription termination at each of the poly(A) addition sites or by post-transcriptional cleavage of a larger precursor transcript to reveal the correct 3′ termini. Transcription beyond the poly(A) addition site(s), with subsequent cleavage of the primary transcript and polyadenylation, has been demonstrated in other systems, such as β-globin,[18–20] SV40 late,[21,22] and adenovirus early and major late[23–25] transcription units. In the case of vimentin RNA processing, it is also possible that a transcript that is cleaved and/or polyadenylated at one of the downstream sites may be further processed, ultimately to yield a 2.0 kb mRNA. Experiments involving rapid labeling and detection of vimentin primary transcripts should help define the limits of the vimentin gene transcription unit and the processing steps necessary to generate the observed 3′ termini.

The role of 3′ untranslated sequences in mRNA is currently unknown. However, in addition to chicken vimentin mRNA, size heterogeneity of 3′ untranslated regions is also observed in mRNAs coding for mouse dihydrofolate reductase (four mRNAs),[26] α-amylase (two mRNAs),[27] β_2-microglobulin (two mRNAs),[28] and eel calmodulin (at least two of three mRNAs).[29] The cell- and tissue-specific expression of the vimentin mRNA species implies that variations in the lengths of the 3′ untranslated region are functionally significant. Perhaps these sequences affect the subcellular compartmentation of the mRNAs, the relative stability of the two messages, or both. In this regard, it is puzzling that mammalian cells express only one vimentin mRNA species[30,31] whose 3′ untranslated sequences are highly homologous (83%) to those of the chicken vimentin mRNA;[31] the mammalian mRNA's 3′ terminus appears to coincide with a position near the second polyadenylation signal of the chicken molecule.[31] Any explanation for the importance of cell-specific expression of two mRNAs with different 3′ untranslated regions therefore must take into account the presence of only one vimentin mRNA in mammals.

Vimentin Gene Expression in Chicken Embryonic Erythroid Cells

As described above and shown in FIGURE 1, circulating erythroid cells from 10- and 15-day chick embryos express predominantly the 2.0 kb vimentin mRNA, whereas erythroid cells from 4-day-old embryos express both mRNA classes.[13] Furthermore, the levels of vimentin mRNA in 10- and 15-day cells are ~20 and ~50 times higher than in 4-day cells, respectively. Circulating blood cells from 4-day-old embryos are a mixture of early and late polychromatophilic primitive erythroblasts, whereas cells from 10-day-old embryos are ~25% primitive cells, ~35% mid to late polychro-

matophilic definitive erythroblasts, and ~35% mature definitive erythrocytes.[32] At 15 days of embryonic development, 60-70% of the erythroid cells are mature definitive erythrocytes, with late polychromatophilic erythroblasts making up the remaining fraction.[32] Since primitive series and definitive series erythroid cells represent different cell lineages, the accumulation of the 2.0 kb vimentin mRNA between 4 and 10 days of development should be considered to be lineage specific. Only a slight proportion of vimentin mRNA from 10- and 15-day cells can possibly derive from primitive series cells, as these cells represent a small percentage of the 10-day population and are virtually absent in 15-day-old embryo blood. Hence, the ~twofold increase in erythroid vimentin mRNA levels observed between 10 and 15 days of embryogenesis can be ascribed to an induction of expression solely within the definitive series lineage.

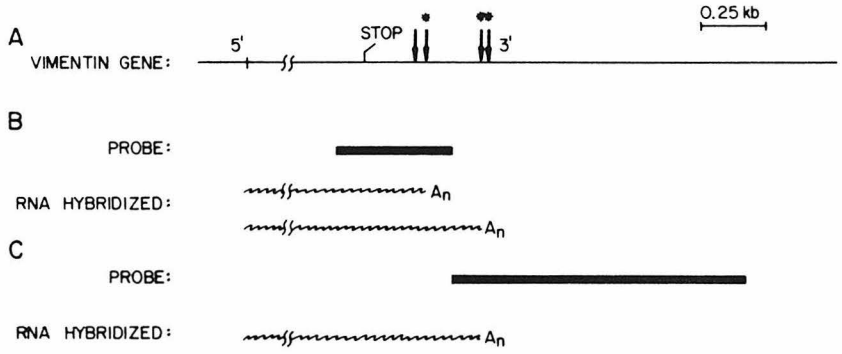

FIGURE 2. Variations in the lengths at the 3′ end are responsible for the size differences of the chicken vimentin mRNAs. Schematic representation of the vimentin gene, showing the positions of the stop codon (STOP) and the putative poly(A) addition signals (5′-AATAAA-3′) (arrows) as determined by DNA sequencing(A).[16] Asterisks denote the poly(A) addition sites, which are utilized in chick embryonic skeletal muscle.[17] When a ^{32}P-labeled probe, which includes amino acid coding sequences as well as 3′-untranslated sequences, is hybridized to RNA containing both vimentin mRNAs, both the 2.0 kb and 2.3 kb species are detected(B).[13] However, a ^{32}P-labeled probe derived from the 3′ end of the gene only hybridizes to the 2.3 kb RNA(C).[13]

The increase in vimentin mRNA abundances during chicken embryonic erythropoiesis appears to underlie the pattern of vimentin protein expression. In 4-day embryos, primitive series cells synthesize low levels of vimentin (Blikstad, I. and Lazarides, E., unpublished results). In contrast, blood cells from 10- and 15-day embryos display an increased level of vimentin synthesis and vimentin filament accumulation.[33,34] The expression of vimentin filaments therefore appears to be regulated primarily transcriptionally or by RNA stabilization.

The factors that regulate both the induction and selection of the 2.0 kb vimentin mRNA in definitive versus primitive cells remain to be elucidated. Different hemopoietic microenvironments may influence the expression of the vimentin gene. The pattern and level of expression also may be genetically determined within each erythroid lineage. Further studies using more defined populations of cells and cultured cell systems[35,36] should help delineate the factors relevant to this phenomenon.

Repression of Vimentin Gene Expression during Mammalian Erythropoiesis in Vitro

The dramatic change of vimentin mRNA levels during chicken embryonic erythropoiesis led us to search for a well defined culture system in which to study the regulation of vimentin gene expression during differentiation. For over a decade, Friend murine erythroleukemia (MEL) cells have provided an excellent model system for studying the molecular events in mammalian erythropoiesis.[37] Chemical induction of MEL cell differentiation evokes a series of events that resemble those occurring in mammalian erythropoiesis *in vivo,* such as a change from a basophilic erythroblastic to an orthochromatophilic normoblastic phenotype,[38] cessation of cell proliferation,[39,40] induction of heme synthetic enzyme activities,[41,42] elevation in iron uptake and heme synthesis,[38] induction of globin mRNAs,[43–45] and accumulation of hemoglobin.[38] A variety of membrane-associated alterations are also induced, including changes in surface antigen expression,[46–48] an increase in transferrin receptor levels,[49] induction of the membrane skeleton protein spectrin[46,50] and the transmembrane anion transporter,[51] and a decrease in Na,K (ATPase) activity.[52,53] We therefore sought to determine if any changes in vimentin expression occur during MEL cell maturation, and if so, to establish where in the developmental program such changes may reside.

We have found that in striking contrast to the pattern of vimentin expression in chicken erythroid cells, MEL cells rapidly repress the expression of vimentin upon induction with dimethyl sulfoxide (DMSO),[14] a potent inducer of MEL cell differentiation.[38] Immunofluorescence microscopy using an anti-vimentin antiserum shows that vimentin filaments are present in untreated MEL cells, but are lost rapidly upon induction with DMSO[14] (FIGURE 3). [^{35}S]methionine labeling experiments have revealed that synthesis and assembly of cytoskeletal vimentin is significantly reduced by 24 hr of induction.[14] Hence, the loss of vimentin filaments from differentiating MEL cells may be facilitated by a shutdown of vimentin synthesis with a concomitant dilution of existing filaments by cytokinesis.

To investigate the mechanisms underlying the decline of vimentin synthesis in differentiating MEL cells, we performed quantitative RNA blots using a chicken vimentin cDNA as probe.[14] In uninduced cells, this cDNA recognizes a single 2.1 kb RNA band[14] that represents the mature vimentin mRNA, as reported by others.[30,31] By 12 hr of DMSO-mediated differentiation, the level of vimentin mRNA falls to

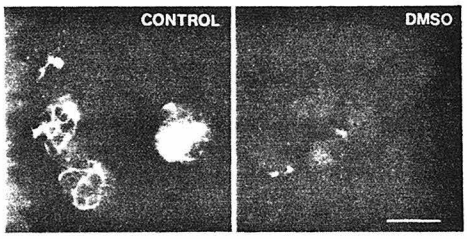

FIGURE 3. Differentiation of MEL cells induced by DMSO causes the loss of vimentin filaments. MEL cells were cultured in the presence of 1.8% DMSO for 72 hr or maintained in control medium, as indicated. Cells were prepared for indirect immunofluorescence microscopy using an anti-vimentin antiserum as described previously.[14] Bar, 10 μm.

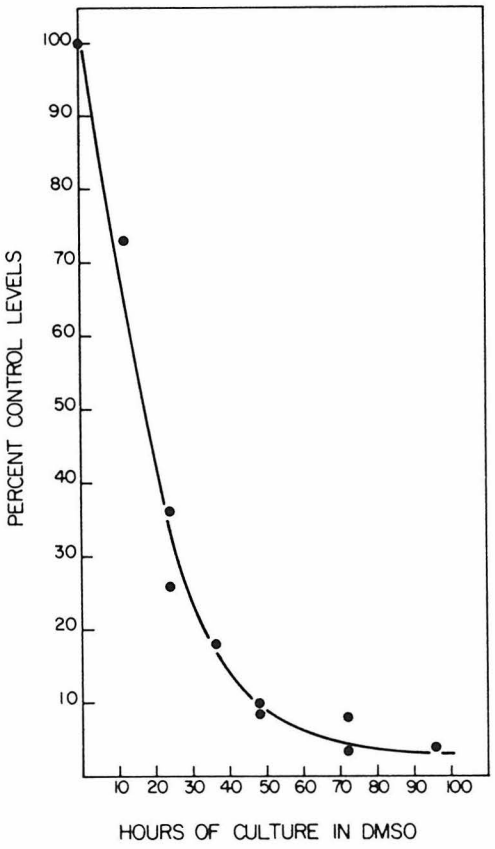

FIGURE 4. Vimentin mRNA levels during MEL cell differentiation. Poly(A)$^+$ RNA was prepared from cells cultured for various periods in the presence of 1.8% DMSO or in control medium, and subjected to quantitative RNA blot analysis using a vimentin-specific probe.[14] Autoradiograms were scanned and peak areas were determined. The values shown are normalized to total cellular poly(A)$^+$ RNA content and are expressed as a percentage of the control levels (100%).

~70% of control amounts and rapidly thereafter to ~30% at 24 hr, ~10% at 48 hr, and ~4% at 96 hr[14] (FIGURE 4). For comparison, a significant increase in β-globin mRNA accumulation does not occur in these cells until 24-36 hr of differentiation.[14] The repression of vimentin mRNA levels in DMSO-treated MEL cells therefore represents an early and rapid process in this developmental program. Moreover, our results demonstrate that, as in chicken erythroid cells, vimentin filament expression in this system is regulated by the abundance of vimentin mRNA.

Although the precipitous decrease in vimentin mRNA occurs early in MEL cell differentiation, this event appears to be associated with the subsequent commitment to terminal differentiation.[14] Commitment to terminal differentiation represents the capacity of MEL cells to continue irreversibly in their differentiation program in the absence of chemical inducer.[39,40] During induction by DMSO, a significant percentage of committed cells appears in the population after a lag period of 20-24 hr, and the number of committed cells attains a plateau level by ~48 hr.[39] From FIGURE 4 we see that the decline in vimentin mRNA levels precedes the major onset of commitment; by 24 hr of induction, vimentin mRNA levels have fallen ~threefold, and ~10-fold at 48 hr. Hexamethylenebisacetamide (HMBA), another potent inducer of MEL cell terminal differentiation,[54] like DMSO also causes a dramatic reduction in vimentin

mRNA levels.[14] On the other hand, hemin, an agent that effects a rapid increase in globin mRNA levels,[45,55,56] but does not induce other events characteristic of terminal differentiation,[56] causes only a ~twofold reduction in vimentin mRNA.[14] Together these results suggest that repression of vimentin mRNA expression is a component of the MEL cell terminal differentiation program, although the decline temporally precedes commitment.

It should be noted that we have not assayed for commitment in our MEL cell culture system. However, the kinetics of globin induction we observed[14] are similar to those found in the studies that did assay for commitment.[39] In the latter studies, globin accumulation appeared to parallel or slightly precede the onset of commitment.[39] Hence, given that vimentin mRNA levels begin to decrease significantly by 12 hr, we feel confident that this event indeed precedes absolute commitment.

Our interpretation that the changes in vimentin expression are associated with MEL cell commitment is consistent with observations that indicate that development of the committed phenotype is a multistep process. A multistep process is demonstrated by the growth of mixed benzidine-positive and -negative colonies during cloning in the absence of inducer.[39] The existence of mixed colonies indicates that a series of events can occur subsequent to and distinct from earlier events that were evoked in the presence of inducer. Experiments using inhibitors of MEL cell terminal differentiation have further shown the multistep nature of the acquisition of commitment.[57] A definitive answer regarding the relationship between vimentin expression and commitment can be established by studying the effects of inhibitors of MEL cell differentiation[58–67] and the use of hypersensitive or non-inducible MEL cell lines.[68]

Vimentin Expression in Avian and Mammalian Erythropoiesis: Functional Implications and Future Prospects

The biological function(s) of intermediate filaments remains elusive. However, a structural role for these filaments has been inferred from morphological observations.[69,70] For example, nucleated chicken erythrocytes possess a vimentin filament network that attaches to the plasma membrane, spans the cytoplasm, and appears to anchor the centrally located nucleus.[34] In contrast, intermediate filaments have never been observed in the mature, anucleate mammalian erythrocyte. Furthermore, Dellagi and co-workers have shown by immunofluorescence microscopy that vimentin expression is lost during the erythroblastic stages of human erythropoiesis *in vivo*.[71] Although this loss of vimentin could not be correlated with a particular stage in differentiation,[71] our studies utilizing MEL cells have demonstrated that the disappearance of vimentin filaments is a rapidly inducible event during erythropoiesis *in vitro*[14] (FIGURES 3 and 4).

Considering the different patterns of vimentin expression observed in chicken and mammalian erythroid development, it is possible that enucleation during mammalian erythropoiesis is facilitated by the loss of vimentin filaments. The cessation of vimentin synthesis during mammalian erythropoiesis may allow the dilution of existing filaments (by cell division), which otherwise might physically inhibit enucleation.[72] The loss of vimentin filaments during mammalian red cell development also may reflect the changing state of the plasma membrane.[46–53] Indeed, a dramatic morphological change occurs as the round, nucleated erythroblastic cell gives rise to the smaller, biconcave disc-shaped anucleate erythrocyte. In any case, the dynamic positive and negative regulation of the vimentin gene in avian and mammalian erythropoiesis suggests that the presence

or absence of vimentin filaments represents an essential feature in each of these differentiation programs.

MEL cell differentiation provides an excellent model system for investigating the regulation and consequences of vimentin gene expression during erythropoiesis. The similarity in patterns of vimentin expression during mammalian erythropoiesis *in vivo*[71] and *in vitro*[14] strongly suggests that the mechanisms for vimentin gene regulation in MEL cells reflect or are identical to those operative *in vivo*. One limitation of the *in vitro* system, however, is that MEL cells rarely differentiate to or beyond the reticulocyte stage.[73] We are now carrying out experiments involving the transformation of MEL cells with foreign vimentin genes in an attempt to investigate further vimentin gene regulation. Additionally, manipulation of vimentin gene expression may yield valuable information regarding the function of vimentin filaments during red blood cell maturation.

Noncoordinate Regulation of Vimentin and Desmin Genes During Myogenesis in Vitro

Desmin is the intermediate filament subunit that is specifically expressed in skeletal, cardiac, and most smooth muscles.[69,70] Previous studies have shown an induction of desmin synthesis and accumulation upon fusion of chicken myoblasts into multinucleate myotubes during myogenesis *in vitro*.[7] This induction is manifested by the appearance of cytoplasmic desmin-containing filaments.[6,7] During later stages of differentiation, desmin redistributes from a cytoplasmic filamentous pattern to a sarcomeric Z-line association at a time when adjacent sarcomeres become aligned.[6,7] In adult chicken skeletal muscle, desmin is localized at the peripheries of Z-discs.[74] One interpretation of the above observations is that desmin or desmin-containing filaments both facilitate and maintain the cross-striated appearance in skeletal muscle by redistributing to the Z-disc peripheries and interlinking adjacent myofibrils across the myocyte cytoplasm.[7,8,74] Vimentin follows the same redistribution as desmin during myogenesis, but is present in mitotic and post-mitotic myoblasts, as well as in fusing and post-fusion myotubes.[7]

The mechanism by which intermediate filament redistribution occurs during myogenesis is presently unknown. Desmin and vimentin may associate preferentially with Z discs due to the *de novo* appearance of binding sites at these structures. Another possibility involves the developmentally regulated expression of Z-disc-specific desmin and vimentin mRNAs, whose products would only localize at Z discs. Post-translational modifications of desmin and vimentin also may affect their subcellular localizations and interactions. Lastly, the ratio of vimentin to desmin may influence intermediate filament distribution. To distinguish which of the above possibilities are plausible, and to determine the means of regulating the levels of vimentin and desmin in myogenesis, we have analyzed RNA from this system using recombinant DNA clones for vimentin and desmin.[13,15] From FIGURE 5(A), we see that the levels of the 2.4 kb desmin mRNA increase, beginning at 24-36 hr after plating, and eventually level off by 3-4 days of culture.[15] In this system, the major onset of fusion occurs between 24 and 48 hr after plating, and approximately 50% of the nuclei are present in syncytial myotubes at 30 hr. The large induction of desmin mRNA therefore coincides with the observed increase in desmin synthesis.[7] Hence, the specific expression of this intermediate filament protein during and subsequent to fusion appears to be regulated primarily at the mRNA level. The induction of desmin mRNA levels and

desmin synthesis parallels the induction of other muscle-specific mRNAs (with the concomitant increase in synthesis of these proteins) at the time of fusion.[75-77]

Neither the amount nor the size of the desmin mRNA changes significantly between 4 and 8 days of culture, a time at which the rearrangement of intermediate filaments is observed.[6,7] Since the desmin gene appears to exist as a single copy in the haploid chicken genome,[15] it therefore seems unlikely that a switching of desmin mRNAs

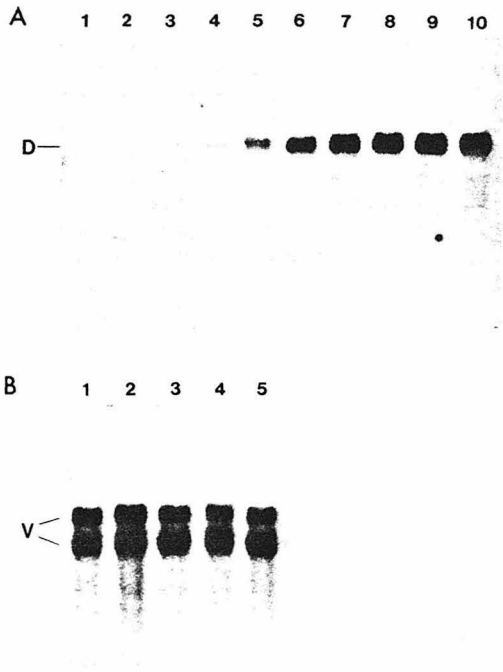

FIGURE 5. RNA blot analysis of desmin and vimentin mRNA levels during myogenesis *in vitro*. (A) Total RNA was isolated from cultured chick myogenic cells, subjected to electrophoresis, blotted to nitrocellulose and hybridized to a ^{32}P-labeled desmin cDNA probe.[15] Lane 1, RNA obtained from cells 6 hr after plating; lane 2, 12 hr; lane 3, 24 hr; lane 4, 36 hr; lane 5, 48 hr; lane 6, 60 hr; lane 7, 84 hr; lane 8, 108 hr; lane 9, 132 hr; lane 10, 192 hr. D indicates the position of the 2.4 kb desmin mRNA band. (B) An RNA blot similar to the one shown in (A) hybridized to a ^{32}P-labeled vimentin cDNA probe. Lane 1, RNA from cells 6 hr after plating; lane 2, 12 hr; lane 3, 24 hr; lane 4, 48 hr; lane 5, 64 hr. Vimentin mRNA levels remain essentially constant through 8 days (192 hr) of culture (data not shown). V denotes the positions of the 2.0 kb and 2.3 kb vimentin mRNAs.

occurs. However, we cannot rule out the possibility that desmin transcripts are differentially processed to yield distinct mRNAs with indistinguishable electrophoretic mobilities. Structural analysis of the desmin gene and its transcript(s) will render a definitive answer to this possibility.

As the single vimentin gene gives rise to two mRNAs with the same amino acid coding region,[13,16,17] the redistribution of vimentin-containing filaments cannot be

caused by the appearance of a Z-disc-specific vimentin gene product.[13] Both vimentin mRNAs are present throughout myogenesis, and there is a slight increase in the amount of the 2.0 kb RNA relative to the 2.3 kb RNA as differentiation proceeds (FIGURE 5, B). At present, we cannot ascribe any importance to this latter observation. Our analysis of vimentin mRNA during myogenesis indicates that there is no significant change in its abundance through 8 days of culture (FIGURE 5, B and unpublished results). This result contrasts with the observed two- to threefold increase in vimentin synthesis that we have reported previously.[7] The discrepancy may be due to differences in standardization (normalization to total RNA versus total protein synthesis) or variations in culturing conditions. Zehner and Paterson have observed a ~threefold increase in vimentin mRNA during myogenesis,[17] but this difference again may be due to dissimilar methods of culture. Nevertheless, it is clear that the genes coding for desmin and vimentin are noncoordinately regulated during myogenesis *in vitro.*

The relatively constant levels of vimentin and desmin expression during the reorganization of intermediate filaments (at ~8 days) renders unlikely the possibility that changing ratios of subunit composition affect localization. By default, we are left with the conclusion that the transition of vimentin- and desmin-containing filaments to Z discs is effected either by the appearance of specific Z-disc receptors or filament-binding proteins, or by modification of the subunits themselves. Both vimentin and desmin are substrates for cAMP-dependent protein kinases,[78,79] and at the time of filament redistribution, the sensitivities and sites of phosphorylation for these kinases change.[79] Furthermore, Danto and Fischman have characterized a monoclonal antibody that recognizes desmin in adult cardiac and skeletal muscle, but not in cultured cardiac muscle cells or certain regions of embryonic myocardium.[80] The differential reactivity to this antibody presumably reflects conformational differences or differential masking, which only allows this epitope to be reactive in adult muscle.[80] The roles of covalent modifications (such as phosphorylation) or protein-protein interactions in this process remain to be elucidated.

Generalized Model for Intermediate Filament Protein Expression

The data discussed in this review show that the expression of vimentin and desmin filaments is regulated primarily at the levels of transcription or RNA stabilization, and not at the level of translation.[13-15] In the case of vimentin, a dramatic change in vimentin mRNA levels during chicken embryonic erythropoiesis is correlated with a similar change at the protein level.[13] The loss of vimentin filaments in differentiating MEL cells also occurs as a result of a rapid and extensive decline in vimentin mRNA.[14] During myogenesis *in vitro,* the rates of desmin synthesis and accumulation increase significantly upon fusion, whereas vimentin levels change to a lesser degree;[7] these alterations in expression are correlated with similar changes in the respective mRNA abundances.[13,15] The large change in magnitude of vimentin mRNA levels during erythropoiesis and desmin mRNA levels during myogenesis suggest similar changes in transcription rates of these genes in the systems studied. However, the effects of variable RNA turnover, as well as transcription, can only be ascertained definitively by direct measurement of these two processes.

During terminal differentiation of certain cells, vimentin may be partially or completely replaced by the cell type-specific subunit. The regulation of vimentin expression in MEL cells by mRNA abundance suggests a similar type of control for vimentin during the replacement of vimentin by specific intermediate filament subunits in other

differentiating cells. Our findings concerning vimentin and desmin regulation during myogenesis further suggest that the ratios of intermediate filament subunits within a cell reflect the ratios of the respective mRNAs. This is further supported by the observations that relative abundances of vimentin correlate with the levels of mRNA in different tissues,[13] and desmin mRNA is present only in tissues or cells that express the desmin polypeptide.[15] Similarly, we have shown by RNA blot analysis using a GFAP cDNA probe that GFAP mRNA is present in the central nervous system, but is not detectable in any of the non-nervous tissues examined.[15] The differential expression of keratin polypeptides in various epithelial cells has also been shown to be due to differences at the mRNA level.[81,82] We extrapolate the results discussed above to conclude that in general, the expression of intermediate filament subunits is determined by the presence and abundance of the corresponding mRNAs. A notable exception is presented by mouse squamous cell carcinomas, wherein the mRNA coding for a 67,000 dalton keratin is specifically masked, preventing its expression.[83] Determination of the prevalence of mRNA masking in the regulation of intermediate filament protein expression awaits further experimentation using other cell types.

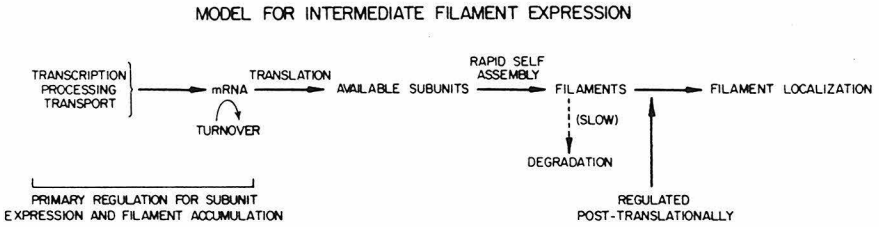

FIGURE 6. A general model for the regulation of intermediate filament expression. Rapid self-assembly of intermediate filaments is facilitated by the availability of soluble subunits. The availability of subunits in turn is determined primarily at the mRNA level. The intracellular localization of filaments may be influenced by post-translational modification of the subunits themselves or by protein-protein interactions (see text).

The presence of intermediate filaments comprising two different subunits has been observed in a number of systems. Chemical cross-linking studies have demonstrated that intermediate filament heteropolymers occur *in vivo* and that non-identical subunits can exist as nearest neighbors.[84,85] Reconstitution studies have also shown that vimentin and desmin can assemble to form heteropolymers *in vitro*.[86] A uniformly sparse decoration of intermediate filaments in chicken erythrocytes with an anti-neurofilament (NF70) antibody has been observed by immunoelectron microscopy;[12] these cells contain substoichiometric amounts of NF70 as compared to vimentin.[12] The simplest explanation for these results is that the observed antibody decoration reflects the random co-polymerization of NF70 and vimentin.[12] Similarly, there is a punctate distribution of desmin in intermediate filaments of fibroblasts,[87] where only a very low amount of desmin is present.[88] Studies from this laboratory have demonstrated the rapid post-translational assembly of vimentin filaments from a saturable pool of soluble vimentin in chicken erythroid cells.[33,89] The kinetics of assembly suggest that the formation of filaments within the cell is determined primarily by the availability of soluble subunits and is not regulated or limited post-translationally.[33,89] The formation of heteropolymers with a random distribution of subunits is consistent with the interpretation that assembly of intermediate filaments is dependent mainly on the availa-

bility of the subunits themselves. However, the existence of microdomains of homologous subunits within a co-polymer is neither demonstrated nor ruled out by the above experiments. It is possible that subunits are incorporated into growing filaments as small oligomeric homopolymers, which would appear to be soluble by conventional fractionation techniques.[33,89]

The availability of intermediate filament subunits for assembly appears to be regulated transcriptionally or post-transcriptionally, but not translationally (with the above noted exception[83]) or post-translationally.[13–15,33,89] Since intermediate filaments have been shown to be stable cellular constituents,[90] we propose the general conclusion that regulation of intermediate filament subunit abundances at the mRNA level directly regulates the expression of intermediate filaments (FIGURE 6). Although the major point of regulation appears to precede translation, we cannot discount other influences on intermediate filament expression. Such influences include those that determine the localization of filaments within the cell. For example, some post-translational (e.g. phosphorylation) or allosteric mechanism must exist to alter the form and localization that desmin and vimentin filaments assume during their redistribution during myogenesis.[13,15,79] Other subtle translational and/or post-translational mechanisms may exist to fine-tune the assembly and the ultimate expression of intermediate filaments.

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