Gene Expression in Visceral Endoderm: a Comparison of Mutant and Wild-type F9 Embryonal Carcinoma Cell Differentiation

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Abstract. We have examined the abundance and cell specificity of several mRNAs that are regulated during the retinoic acid (RA)-induced differentiation of F9 embryonal carcinoma cells to visceral endoderm. The experiments confirmed the multistep nature of this process by demonstrating the expression of the ERA-1/Hox 1.6 message within 6 h after RA addition; the expression of messages specific for the extracellular matrix proteins laminin B1 and B2, and collagen IV(α1) between days 4 and 12; and the expression of two visceral endoderm markers, α-fetoprotein (AFP) and H19, by days 8-15. In situ hybridization experiments revealed that the collagen IV(α1) mRNA is restricted to the outer cell layer of F9 cell aggregates regardless of the presence or absence of RA. Laminin B1 and B2 mRNAs are concentrated in the outer cell layer of RA-treated aggregates although significant levels of message are also observed within the interior cells of the aggregates. Unexpectedly, AFP mRNA is detectable in only a subset of the outer cells of F9 cell aggregates grown 15 d in the presence of RA.

The results obtained from wild-type F9 cells were compared with those from a mutant F9 cell line, RA-5-1, which was previously shown to synthesize collagen IV containing six- to ninefold less 4-hydroxyproline than that in wild-type F9 cells. RA-5-1 cells exhibit four- to sixfold less of the mRNAs encoding two visceral endoderm proteins, AFP and H19, than wild-type F9 cells after RA treatment of RA-5-1 aggregates. RA-5-1 cells, however, do exhibit an RA-associated increase in the level of ERA-1/Hox 1.6 mRNA within 6 h after adding RA. Although the collagen IV protein level is similar in wild-type F9 and RA-5-1 aggregates, the collagen IV(α1) message level is 6-20-fold greater in aggregates of mutant cells than in aggregates of wild-type cells. Moreover, in situ hybridizations showed that this message is evenly distributed throughout the RA-5-1 aggregates rather than restricted to the outer cell layers as it is in wild-type F9 aggregates. These results suggest that abnormal collagen IV expression and localization are associated with decreased expression of the visceral endoderm markers, AFP and H19, in RA-5-1 cell aggregates.

T HE first cell types to differentiate from the inner cell mass of the mouse blastocyst are the parietal and visceral endoderm (VE).[1] In the developing mouse embryo, VE lines the inner cell mass and eventually forms the visceral yolk-sac, while parietal endoderm cells migrate onto the inner surface of the trophectoderm and lay down a thick extracellular matrix (ECM) known as Reichert's membrane. While the question of how a subset of undifferentiated cells becomes the highly specialized extraembryonic endoderm is of great interest, the small size and inaccessibility of the mouse embryo makes the in vivo study of this problem difficult. Fortunately, the murine embryonal carcinoma cell line F9 can be induced to differentiate into cells resembling either parietal endoderm or VE in vitro, thus facilitating the study of this developmental question.

F9 embryonal carcinoma cells are the malignant stem cells of a mouse teratocarcinoma. Monolayer cultures of F9 embryonal carcinoma cells grown in the presence of the vitamin A derivative, retinoic acid (RA), differentiate into nonmalignant cells resembling the primitive endoderm of the mouse blastocyst. If the media is further supplemented with dibutyryl cAMP and theophylline, the cells become biochemically indistinguishable from parietal endoderm. As in the mouse embryo, these cells are characterized by the increased production of numerous proteins including the ECM proteins laminin and collagen IV (Strickland et al., 1980). One of the earliest known molecular responses to RA is an increase in the abundance of a Hox 1.6 homeobox containing transcript (LaRosa and Gudas, 1988b). The induction of the ERA-1/Hox 1.6[2] mRNA within 2 h after RA treatment of F9

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1. *Abbreviations used in this paper:* AFP, α-fetoprotein; ECM, extracellular matrix; RA, retinoic acid; VE, visceral endoderm.

2. The gene referred to as ERA-1 in LaRosa and Gudas (1988a) was subsequently shown to contain the Hox 1.6 homeobox sequence (Baron et al., 1987; LaRosa and Gudas, 1988b) and will be referred to as ERA-1/Hox 1.6 in this paper.

cell monolayer cultures has been shown to be independent of protein synthesis, suggesting that this gene is a primary target of RA (LaRosa and Gudas, 1988a).

Alternatively, F9 cells grown in suspension in the presence of RA aggregate and, within 1 wk, form embryoid bodies consisting of stem cells surrounded by an outer layer of cells that resemble mouse VE (Hogan et al., 1981). The differentiation of F9 stem cells into VE has been shown to be a multistep process of which some crucial features are aggregation, epithelium and basement membrane formation, and finally secretion of VE-specific differentiation products such as α-fetoprotein (AFP) and H19 (Grover et al., 1983b; Pachnis et al., 1984). These messages, which are coordinately regulated during murine embryogenesis (Pachnis et al., 1984), may be detected in RA-treated F9 embryoid bodies by 5 d of culture (Grover et al., 1983a; Young and Tilghman, 1984). The importance of a contiguous basement membrane in attaining maximal secretion of AFP was demonstrated by the inability of F9 stem cells to differentiate into VE when subjected to experimental treatments that alter basement membrane formation, such as enrichment or depletion of exogenous laminin (Grover et al., 1983a) or tunicamycin treatment (Grabel and Martin, 1983).

To analyze further the role of the ECM in the formation of VE, we have characterized the differentiation of a mutant F9 cell line RA-5-1. Monolayer cultures of RA-5-1 cells exhibit a six- to ninefold reduction in the posttranslational hydroxylation of collagen IV, consistent with the observation that the RA-5-1 prolyl-4-hydroxylase has an abnormally high K_m for collagen IV hydroxylation (Wang and Gudas, 1984; Wang et al., 1989). The RA-5-1 line was isolated by virtue of its ability to grow in soft agar in the presence of RA; wild-type F9 cells differentiate in the presence of RA and are no longer able to grow in soft agar. The RA-5-1 cells only partially differentiate into parietal endoderm in monolayer culture, but whether the abnormal collagen IV synthesis also inhibited differentiation of the cells into VE was unknown.

We first characterized the expression and localization patterns of several gene transcripts whose abundance is regulated during differentiation of wild-type F9 cells into VE. The deposition patterns for many of these proteins has been previously described (Grover et al., 1983b; Grabel and Casanova, 1986); however, since the ECM proteins and AFP are secreted, these immunocytochemical studies at the light level do not allow definition of the cells synthesizing these proteins. Consequently, we used in situ hybridization to cellular RNA, since mRNA localization clearly identifies the cells in the aggregates that express these genes. A comparison of these results and those obtained from the mutant cell line RA-5-1 indicates that, in addition to its inability to differentiate fully into parietal endoderm, this cell line is unable to differentiate fully into VE. Moreover, the regulation of collagen IV(α1) message levels is perturbed in this cell line. The data presented here suggest that abnormal expression of collagen IV may prevent normal differentiation of F9 embryonal carcinoma cells into VE.

Materials and Methods

Cell Culture and Differentiation

F9 cells were cultured and induced to differentiate into primitive endoderm as described (LaRosa and Gudas, 1988a). To induce VE formation, F9 cells

were dispersed with trypsin into single cells and cultured as aggregates in untreated Petri plates at a density of 1×10^5 cells/ml in the presence of 50 nM RA. The media from the suspended cultures was replaced by fresh media and RA every 48 h.

The F9 mutant stem cell line SYW-F9-M-RA-5-1 was isolated after mutagenesis of F9 wild-type stem cells by exposure for 4 h to 5 μg/ml of N-methyl-N'-nitronitroso-guanidine. Surviving cells were grown under nonselective conditions for 7 d to allow phenotypic expression, followed by plating in 0.34% agarose containing RA over a mouse embryo fibroblast feeder layer. From the mutagen-treated population of F9 cells, we obtained mutant lines that formed colonies in agarose in the presence of 5 μM RA at a frequency of $0.5^{-2} \times 10^{-5}$. The frequency of obtaining spontaneous mutants was $<1 \times 10^{-7}$. The RA-5-1 mutant line was then single-cell cloned. The RA-5-1 mutant line is quite stable and has been cultured continuously for 12–15 mo without changes in its biochemical properties. However, we generally thaw new vials of both F9 wild-type stem cells and F9 RA-5-1 mutant stem cells every 4–5 mo, and this was done before the initiation of the experiments described in this report. All of the Northern blots and in situ hybridizations reported here were then repeated with cells thawed from second vials of both the F9 wild-type and RA-5-1 stem cell lines to ensure their validity.
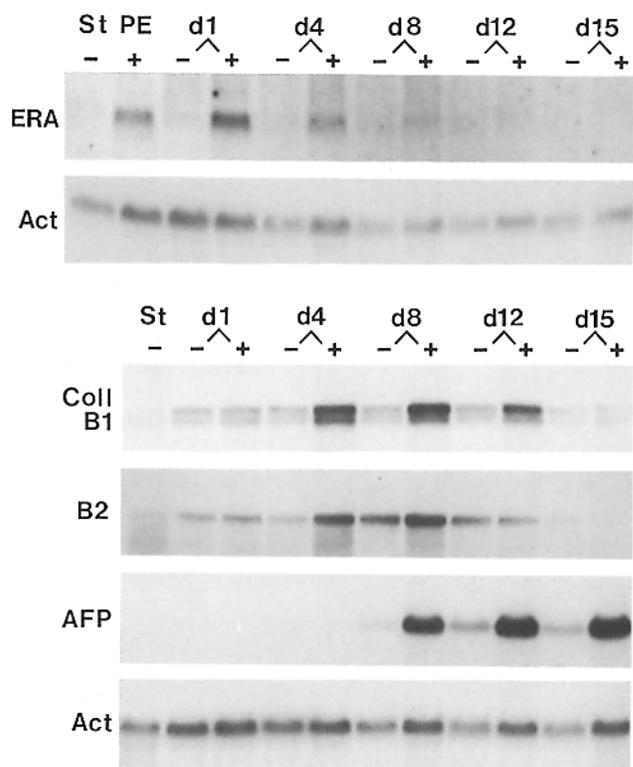

Figure 1. Northern analysis of genes expressed during the differentiation of F9 embryonal carcinoma cells into visceral endoderm. F9 cells were grown in monolayer or suspension culture in the presence (+) or absence (−) of RA. Total cellular RNA was isolated from F9 stem cells (St), F9 primitive endoderm (PE, grown 3 d in monolayer in the presence of 1 μM RA), and aggregates grown 1, 4, 8, 12, and 15 d as indicated, fractionated on agarose/formaldehyde gels, and blotted. ^{32}P-labeled probes were hybridized at the following concentrations: ERA-1, 5×10^6 cpm/ml; collagen IV(α1), laminin B1 and B2, and actin, 10^6 cpm/ml; AFP, 3×10^6 cpm/ml. The RNAs detected are indicated as follows: ERA, ERA/Hox 1.6; ColI, collagen IV(α1); B1, laminin B1; B2, laminin B2; Act, actin, and AFP. The amount of RNA loaded per lane and the exposure time for each Northern blot were as follows: ERA, 8 μg, 2 d with intensifying screen; Act (top), 8 μg, 24 h without a screen; ColI and B1, 4 μg, 2 1/2 d with screen; B2, 10 μg, 18 h with screen; AFP, 10 μg, 2 1/2 d with screen; Act (bottom), 4 μg, 18 h without a screen.
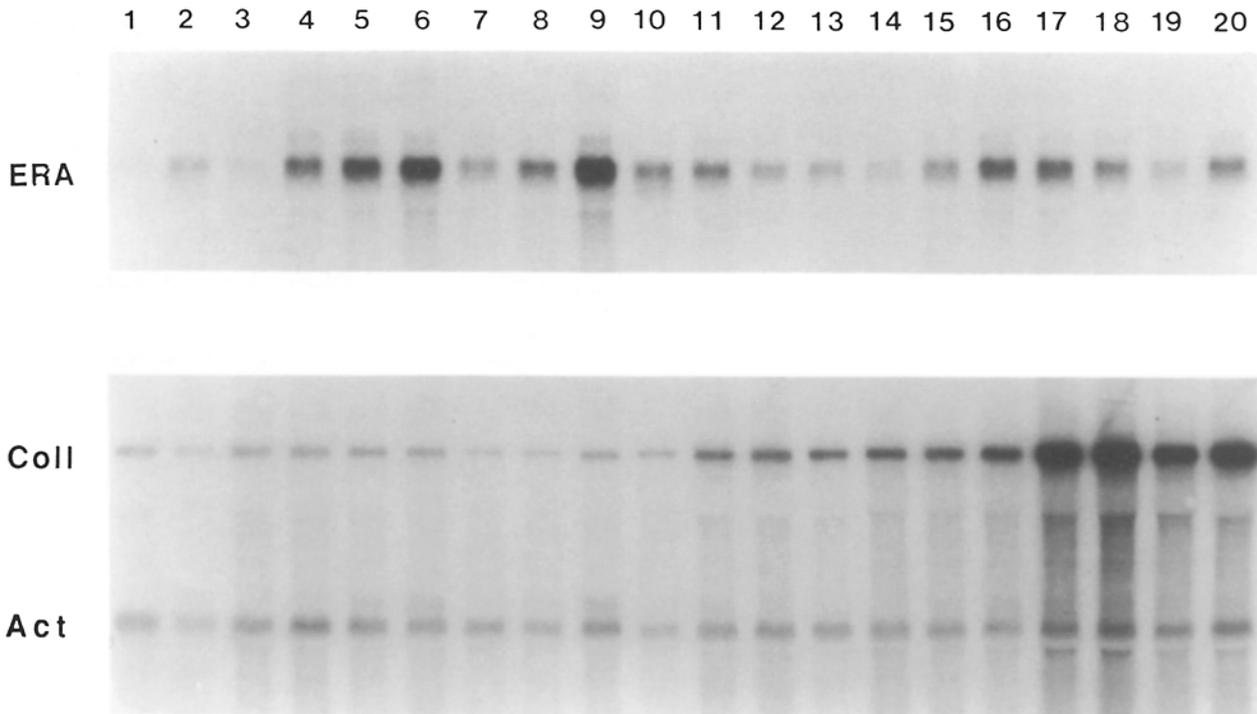
Figure 2. Synthesis of ERA-1/Hox 1.6 and collagen IV(α1) message by RA-5-1 and wild-type F9 cells under different culture conditions. 5 μg of total RNA was isolated from cells treated as described below, fractionated and blotted to nitrocellulose. (Lane *1*) F9 aggregates grown 8 d without RA; (lane 2) F9 aggregates were grown 7 d without RA, and then RA was added and RNA isolated 24 h later; (lanes *3–6*) F9 monolayer cells grown 6 h without RA (lane *3*) or 6 h (lane *4*), 12 h (lane *5*), or 24 h (lane *6*) with 5 × 10⁻⁸ M RA; (lanes *7–13*) F9 aggregates grown 6 h (lane *7*), 12 h (lanes *8*), 24 h (lane *9*), 4 d (lane *10*), 8 d (lane *11*), 12 d (lane *12*), or 15 d (lane *13*) in the presence of 5 × 10⁻⁸ M RA; (lanes *14–20*) RA-5-1 aggregates grown 6 h (lane *14*), 12 h (lane *15*), 24 h (lane *16*), 4 d (lane *17*), 8 d (lane *18*), 12 d (lane *19*), or 15 d (lane *20*) in the presence of 5 × 10⁻⁸ M RA. ^{32}P-labeled probes were hybridized to this blot at the following concentrations: ERA-1/Hox 1.6 (*ERA*), 4 × 10⁶ cpm/ml; collagen IV(α1) (*CoII*), 2.8 × 10⁶ cpm/ml; actin (*Act*), 10⁶ cpm/ml. The exposure time for each panel was as follows: ERA-1/Hox 1.6, 14 h with intensifying screen; and collagen IV(α1) and actin, 42 h without a screen. The results shown here are typical of four independent experiments.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated as described (Wang et al., 1985). RNA was fractionated on 1% agarose/2.2 M formaldehyde gels, blotted, and hybridized as described (LaRosa and Gudas, 1988a).

Plasmids

All cDNA clones employed were of murine origin. pAct-G (Actin) was constructed by subcloning a Pst I fragment from the plasmid pAct-1 (Spiegelman et al., 1983) into pGem-1 (Promega Biotec, Madison, WI). pGem ERA Bam 3 (ERA-1/Hox 1.6) was constructed by subcloning a Bam HI fragment from pERA-1-993 (LaRosa and Gudas, 1988b) into pGem-4-Blue (Promega Biotec). The laminin B1 (pLam389-gem) clone consisted of a 389-bp Pst I fragment subcloned from pcl56 (Wang and Gudas, 1983) into pGem-1. The laminin B2 clone (pLamB2-1.5 gem) contained a 1.5-kb Eco RI fragment of a lambda GT10 phage isolated from a cDNA library constructed in this lab from RNA isolated from F9 cells treated for 8 h with RA, dibutyryl cAMP, and theophylline. The fragment was shown to hybridize to an oligomer specific for the murine laminin B2 sequence (Barlow et al., 1984), and a message of the correct size was observed in RA-treated F9 cells (Gudas et al., 1989). SP65-AFP (sense) and SP65-AFP (antisense) contained a 0.9-kb Pst I fragment from pBR322-AFP2 (Tilghman et al., 1979) subcloned into SP65 (Promega Biotec) in both orientations by Dr. M. Mercola. pBR322-AFP2 and a cDNA clone encoding H19 (Pachnis et al., 1984) were provided by Dr. S. Tilghman. SP64-I5 and SP65-I5 contained a 475-bp Bst E II/Eco RI fragment from pcl5 (collagen IV[α1]; Wang and Gudas, 1983) subcloned into SP64 and SP65 respectively by J. Gold.

Radioactive Probe Synthesis

Single-stranded, [³⁵S]UTP (Amersham Corp., Arlington Heights, IL; 1,000 Ci/mmol) labeled RNA probes (10⁹ cpm/μg) were transcribed from plasmids containing T7 or SP6 promoters as described (Rogers and Zeller, 1989). [³²P]-labeled probes were radiolabeled by the random primer labeling method (Feinberg and Vogelstein, 1984).

In Situ Hybridization to RNA

Aggregates were washed in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 7.2) and dispersed evenly in an equal volume of room temperature OCT compound (Tissue Tek II, Miles Scientific, Naperville, IL). Drops of suspended aggregates were quick-frozen in precooled Freon 23 (Cryoquick, Damon Corp., Needham Heights, MA) in liquid N₂, and stored at −80°C for up to a year. 5–10 μm cryostat sections were made, placed on poly-L-lysine–coated microscope slides and fixed in 4% paraformaldehyde as described in Watkins (1989a). In situ hybridization was performed as described in the alternate protocol of Rogers and Zeller (1989). Autoradiography was performed as described (Rogers, 1989).

Western Blot Analysis

Total cellular proteins were solubilized and electrophoresed on 7.5% polyacrylamide gels as described in Wang and Gudas (1984). The gels were equilibrated and electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) in 0.025 M Tris, 0.0192 M glycine, 20% methanol, and 0.025% SDS, pH 8.3. After transfer, the blots were blocked in 5% milk in PBS, and then incubated in rabbit anti-mouse collagen IV antibody in 5% milk in PBS, followed by ¹²⁵I-labeled protein A.

Immunohistochemistry

Fresh, unfixed 5–10-μm cryosections of aggregates were incubated in rabbit anti-mouse or anti-human collagen IV antisera. Specific binding was revealed by rhodamine-conjugated goat anti-rabbit IgG as described in Watkins (1989b).
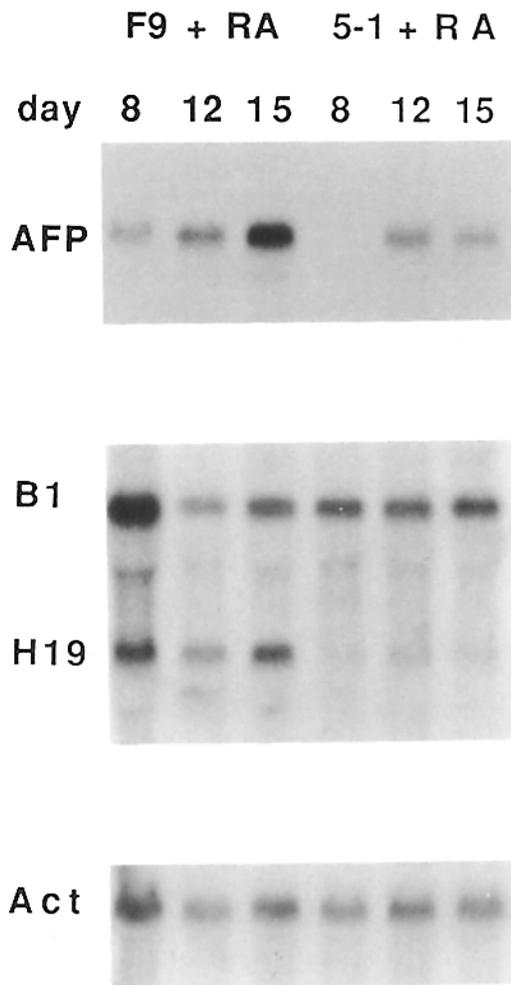
Figure 3. Synthesis of the mRNA for the differentiation markers AFP and H19 by RA-5-1 and wild-type F9 aggregates. Total RNA was isolated from RA-5-1 and wild-type F9 aggregates, 8, 12, and 15 d after the addition of RA as indicated. After agarose/formaldehyde gel fractionation of 10 μg of RNA and blotting, ^{32}P-labeled probes were hybridized sequentially at the following concentrations: AFP, 2.5 × 10⁶ cpm/ml; laminin B1 (B1), 4 × 10⁶ cpm/ml, H19, 4 × 10⁶ cpm/ml; actin (Act), 10⁶ cpm/ml. The exposure times for each panel were as follows: AFP, 41 h with intensifying screen; laminin B1 and H19, 4 1/2 h with two intensifying screens; actin, 42 h without a screen.

Figure 4. In situ hybridization of ^{35}S-labeled antisense AFP probe to 15-d wild-type F9 aggregates. A, B, C, and D show sections of RA-treated aggregates, while E and F show sections of aggregates grown in the absence of RA. Phase micrographs, A, C, E; dark-field micrographs, B, D, F. Exposure time, 13 d. Bar, 25 μm.

Results

Northern Analysis of ERA-1/Hox 1.6 Expression in RA-treated Wild-type F9 Embryoid Bodies

The induction of numerous genes during parietal endoderm formation has been described; however, apart from AFP (Grover et al., 1983a; Young and Tilghman, 1984), studies of VE formation in embryoid bodies have been primarily at the protein level. Thus, we characterized the steady-state transcript levels of several of these genes during F9 embryonal carcinoma cell differentiation into VE using Northern blot analysis. An ERA-1/Hox 1.6 cDNA probe was hybridized to a northern blot of total RNA extracted from F9 aggregates grown for 1, 4, 8, 12, and 15 d in the presence
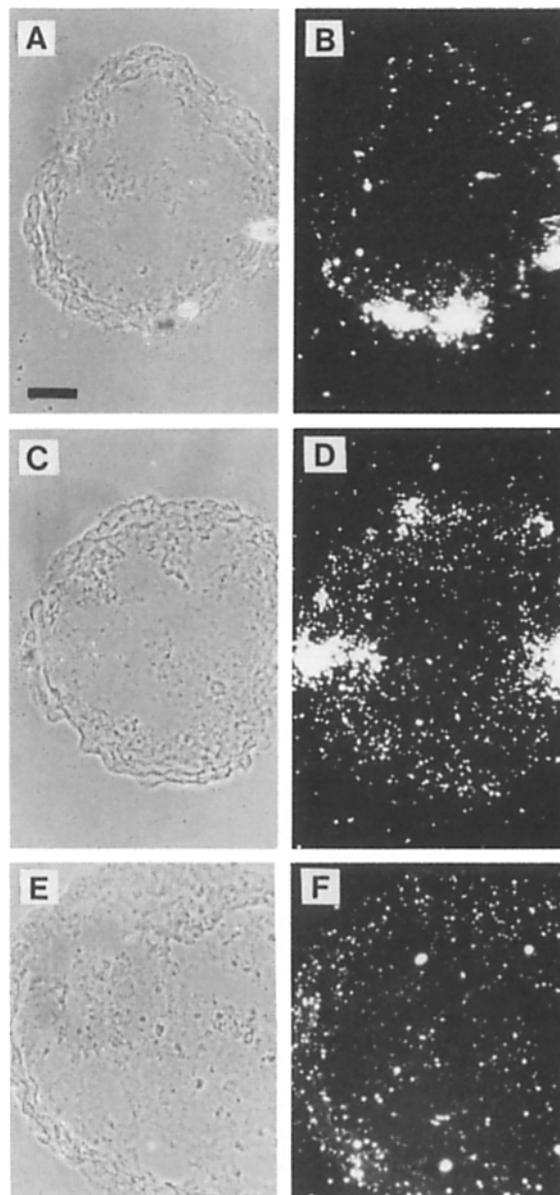
or absence of RA (Fig. 1). A high level of ERA-1/Hox 1.6 mRNA expression was observed on day 1 of aggregate growth in RA containing media, while only a small amount of ERA-1/Hox 1.6 mRNA was observed in the absence of RA. At this point, the aggregates are quite small, many containing about four cells and most no more than eight cells. The early time course of ERA-1/Hox 1.6 mRNA induction by RA is similar whether cells are grown in monolayer or as suspended aggregates (Fig. 2, lanes 3–13). Moreover, the ERA-1/Hox 1.6 message can be increased by the addition of RA to F9 cells grown as aggregates for 7 d in untreated media (Fig. 2, lanes 1 and 2). Thus, the expression of the ERA-1/Hox 1.6 mRNA

is an early, RA dependent molecular event during the differentiation of both parietal and visceral endoderm.

Analysis of the Expression of Laminin B1 and B2, Collagen IV(α1) and AFP mRNAs in Wild-type F9 Aggregates

Since there is evidence that the formation of an organized epithelial layer and basement membrane is required for the expression of the differentiated VE phenotype (Grover et al., 1983a), the abundance of each of three basement membrane protein messages was determined. Laminin B1, laminin B2, and collagen IV(α1) mRNAs were induced by day 4 of aggregate culture, with maximal expression occurring by the eighth day of culture in RA containing media (Fig. 1). These messages subsequently declined and were undetectable by day 15. Although the abundance of these mRNAs was greatest in the presence of RA, some message was detectable in its absence (Fig. 1).

Synthesis of the definitive VE marker, AFP, signals the presence of mature VE. The AFP message was first detected at day 8 of aggregate growth in the presence of RA, and increased in abundance at later times when the basement membrane protein messages declined (Fig. 1). Some AFP message was observed in the absence of RA at later times,

suggesting that a low level of spontaneous VE differentiation occurs. The time course of AFP mRNA expression is similar to that determined by Grover et al. (1983a) and Young and Tilghman (1984) by dot blot hybridization.

Northern Analysis of Gene Expression in RA-5-1 Aggregates

The RA-5-1 mutant was previously shown to be unable to fully differentiate into parietal endoderm. To determine whether or not VE differentiation was affected, RNA isolated from both F9 and RA-5-1 aggregates grown for 8, 12, and 15 d in RA containing media was subjected to Northern blot analysis and hybridized to ^{32}P-labeled probes for the VE markers AFP and H19. The abundance of each message in the RNA extracted from the RA-5-1 cells was four- to sixfold lower than that observed in wild-type F9 cells (Fig. 3). Laminin B1 message levels were similar in mutant and wild-type cells. Thus RA-5-1 cells are unable to fully differentiate into VE or parietal endoderm.

Since it was of interest to determine whether or not RA-5-1 cells exhibit any response to RA, RNA isolated from F9 and RA-5-1 aggregates was probed for the rapidly induced gene ERA-1/Hox 1.6 (Fig. 2). The message was induced within 6 h of growth in RA containing media in both RA-5-1 and
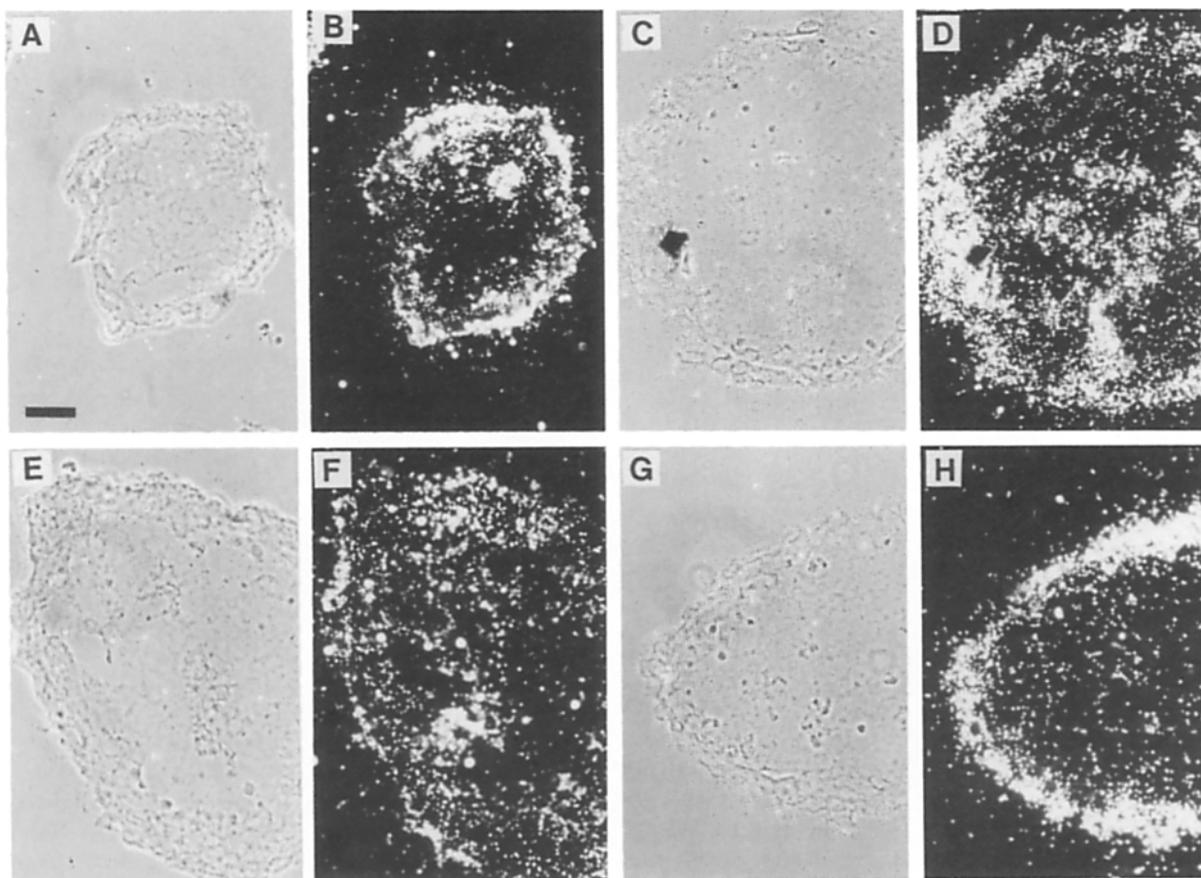

Figure 5. In situ hybridization of ^{35}S-labeled antisense laminin B1 probe to wild-type F9 aggregates. A, B, C, and D show sections of RA-treated aggregates, while E, F, G, and H show sections of aggregates grown in the absence of RA. (A, B, E, and F) Aggregates grown 8 d in culture; (C, D, G, and H) 15 d in culture. (A, C, E, and G) Phase micrographs; (B, D, F, and H) dark-field micrographs. Exposure time, 5 d. Bar, 25 μm.

wild-type F9 cells (compare lanes *7-13* with *14-20*). As in wild-type F9 cells, the ERA-1/Hox 1.6 message was very rare in RA-5-1 cells in the absence of RA (data not shown).

In Situ Hybridization Analysis of AFP, Laminin B1 and B2, and Collagen IV(α1) mRNAs in Wild-type F9 Cell Aggregates

Northern blot hybridization allows quantitation of message abundance but does not allow the localization of the message to specific cell types. For this reason, in situ hybridization to cellular RNA was used to determine first whether the outer VE cell layer of the wild type F9 embryoid bodies synthesize the differentiation products exclusively, and second, whether the collagen IV(α1) mRNA is abnormally localized in RA-5-1 aggregates as well as being overexpressed. In these experiments, antisense transcripts were used to detect the messenger RNA, while their complementary transcripts, sense probes, were used to control for background hybridization.

The location of the AFP transcripts was determined in aggregates grown 15 d with or without RA. This experiment demonstrated that AFP messages are synthesized strongly by only a few of the outer embryoid body cells (Fig. 4, *B* and *D*). Distinct grain clustering was not observed over aggregates grown in the absence of RA (Fig. 4 *F*). Thus, the message for the secreted protein AFP is apparently synthe-

sized by only a small portion of the VE cells of day 15 embryoid bodies.

The location of the RNAs encoding several ECM proteins was determined. Sections of RA-treated and untreated wild-type aggregates were hybridized to ^{35}S-labeled laminin B1 probe. The outer cell layers of embryoid bodies grown for 8 d in RA hybridized strongly to the laminin B1 probe, but the inner stem cells also displayed significant hybridization (Fig. 5 *B*). After 15 d of culture in RA, the pattern was similar (Fig. 5 *D*). The laminin B1 mRNA was distributed more evenly throughout aggregates grown 8 d without RA (Fig. 5 *F*), but by 15 d of growth without RA most grains were restricted to the outer cells (Fig. 5 *H*).

Laminin B2 mRNA was more evenly distributed throughout the embryoid bodies, with the outer layers of day 8 RA-treated embryoid bodies hybridizing most intensely (Fig. 6 *B*). Aggregates cultured for 15 d in RA or for 8 or 15 d without RA displayed a uniform pattern of hybridization to the laminin B2 probe (Fig. 6, *D*, *F*, and *H*).

After hybridization of an antisense probe corresponding to collagen IV(α1) to sections of F9 embryoid bodies grown for 8 d in the presence of RA, silver grains were observed over the outer layer of cells (Fig. 7 *B*). A distinct ring of grains was also observed over aggregates grown in the absence of RA (Fig. 7 *D*). Several additional sections that were hybridized to collagen IV(α1) are shown at lower magnification in Fig. 8. Thus, collagen IV(α1) message appears to be synthe-
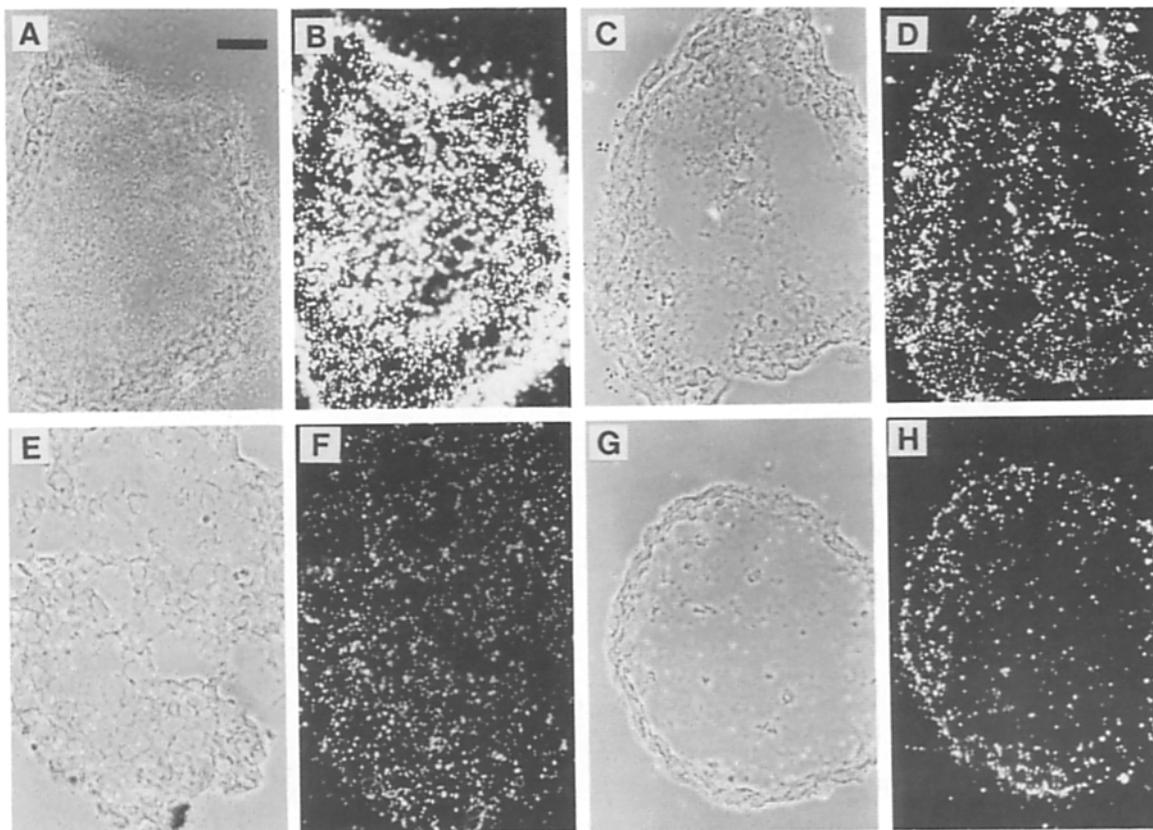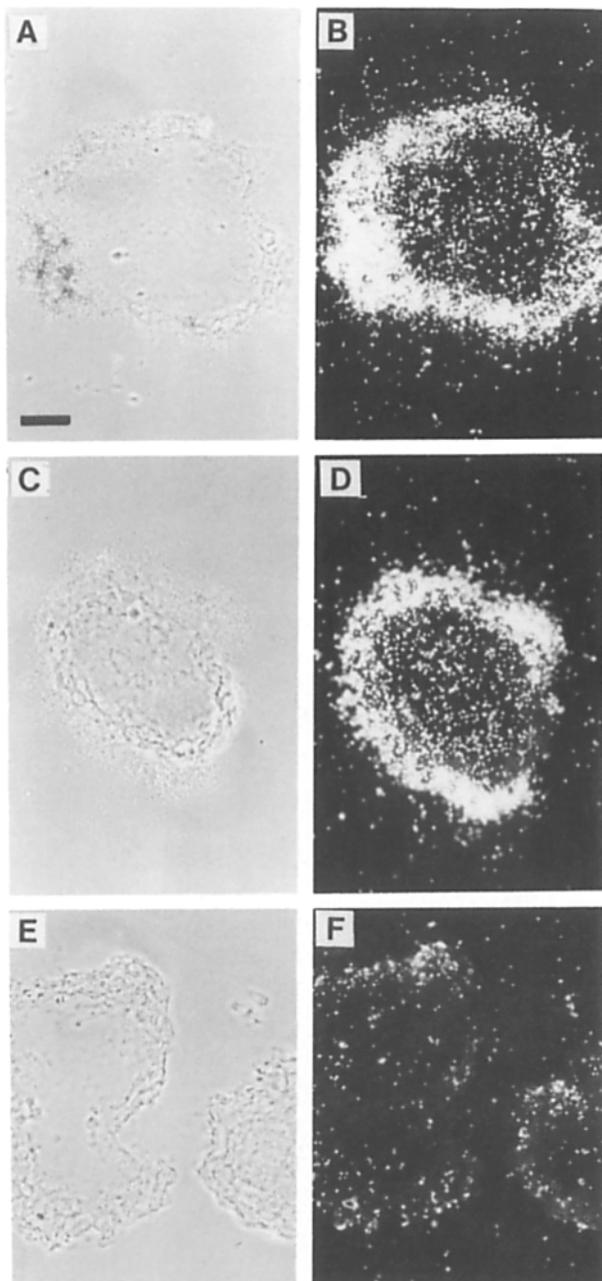

Figure 6. In situ hybridization of ^{35}S-labeled antisense laminin B2 probe to wild-type F9 aggregates. *A, B, C,* and *D* show sections of RA-treated aggregates, while *E, F, G,* and *H* show sections of aggregates grown in the absence of RA. (*A, B, E,* and *F*) Aggregates grown 8 d in culture; (*C, D, G,* and *H*) 15 d in culture. (*A, C, E,* and *G*) Phase micrographs; (*B, D, F,* and *H*) dark-field micrographs. Exposure time, 5 d. Bar, 25 μm.

Figure 7. In situ hybridization of ^{35}S-labeled antisense collagen IV(α1) probe to day 8 wild-type F9 aggregates. *A, B, C,* and *D* show sections hybridized to antisense collagen probe and *E* and *F* show a section hybridized to sense probe. *A, B, E,* and *F* are sections of RA-treated aggregates, while the aggregate shown in *C* and *D* was untreated. (*A, C,* and *E*) Phase micrographs; (*B, D,* and *F*) dark-field micrographs. Exposure time, 3 d. Bar, 25 μm.

parent basement membrane (Fig. 9 *A* and *C*), grain density was uniform over the section, as expected (Fig. 9 *B*). Hybridization of the sense actin probe (Fig. 9 *D*) and all other sense probes (Fig. 7 *F* and data not shown) only resulted in a low background of silver grain accumulation.

In Situ Hybridization of Collagen IV(α1) Probe to RA-5-1 Aggregates

The abundance of collagen IV(α1) mRNA in RA-5-1 aggregates is strikingly higher than that observed in F9 wild-type aggregates (Fig. 2). Therefore, we compared this gene's expression patterns in RA-5-1 and wild-type F9 aggregates by in situ hybridization. The results indicate that not only is the message overexpressed in both RA-treated and untreated RA-5-1 aggregates relative to F9 aggregates, but that the pattern is altered (Fig. 10). All cells, including the inner cells, exhibit high levels of collagen IV(α1) mRNA. In contrast, the pattern of laminin B1 message accumulation is unchanged in RA-5-1 aggregates relative to wild-type F9 aggregates (data not shown).

Characterization of the Collagen IV Protein in RA-5-1 Aggregates

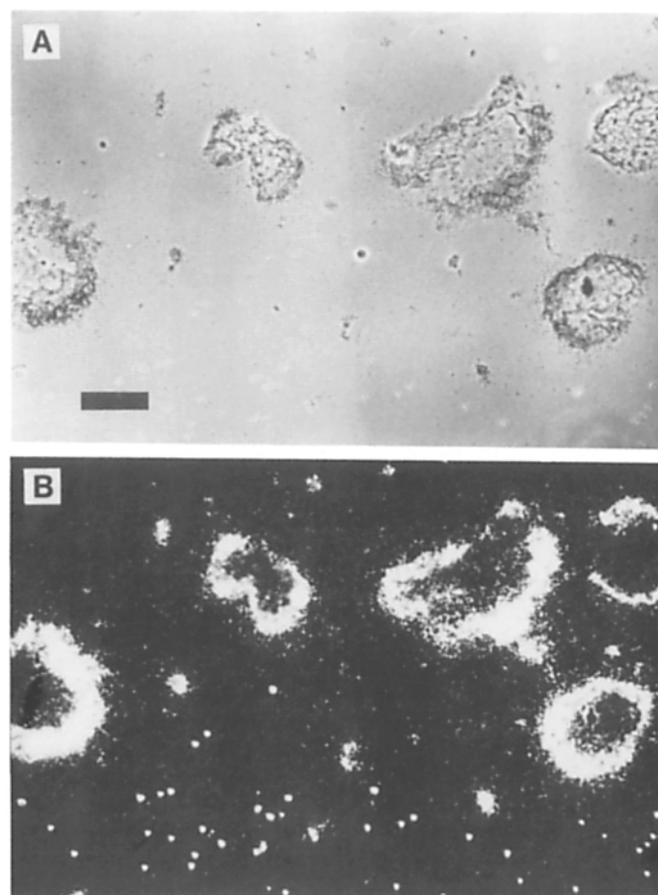Collagen IV secreted from RA-5-1 cells treated with RA,

Figure 8. Low magnification photograph of aggregate sections hybridized with collagen IV(α1) probe. *A* shows a phase micrograph and *B* shows a dark-field micrograph of wild-type F9 aggregates grown 8 d in the absence of RA. Bar, 50 μm.
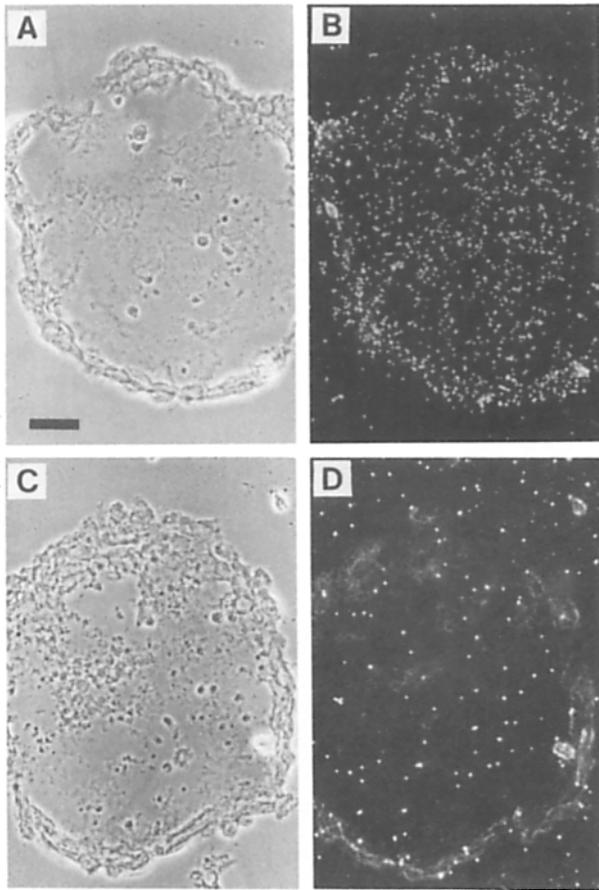
sized exclusively by the outer layer of cells in wild-type aggregates grown 8 d in the presence or absence of RA.

We controlled for artifactual levels of hybridization over the different cell types by hybridizing a radioactive antisense actin probe to embryoid bodies grown 8 d in the presence of RA. We have previously observed that actin mRNA is equally expressed by most cell types (LaRosa and Gudas, 1988a, b; and Figure 1). Although the outer layer of cells was epithelial-like and separated from the inner cells by an ap-

Figure 9. In situ hybridization of ^{35}S-labeled actin probes to wild-type F9 aggregates. ^{35}S-labeled single-stranded RNA probes complementary (antisense, *A* and *B*) or identical (sense, *C* and *D*) to actin mRNA were synthesized and hybridized to sections of aggregates grown 8 d in the presence of RA. After hybridization, slides were exposed to autoradiographic emulsion. Phase micrographs of each section are shown in *A* and *C*, while the corresponding dark-field micrographs are shown in *B* and *D*. In dark field, silver grains are seen as white dots against a black background of unexposed emulsion. Exposure time, 2 d. Bar, 25 μm.

dibutyryl cAMP, and theophylline in monolayer culture was previously shown to be significantly lower in molecular mass than collagen IV secreted from wild-type F9 cells as a result of a six- to ninefold decrease in 4-hydroxyproline content of RA-5-1 derived collagen IV (Wang et al., 1989; and Fig. 11, lanes *B* and *D*). To determine whether the collagen IV protein, as well as the RNA, was overexpressed in the RA-5-1 aggregates, Western blot analysis was performed (Fig. 11). In both amount and molecular weight, collagen IV protein in RA-5-1 aggregates resembled that found in wild-type F9 aggregates (Fig. 11, lanes *E–H*). Moreover, three distinct bands were observed in the lanes containing collagen IV isolated from RA treated aggregates (Fig. 11, lanes *F* and *H*); the slowest migrating band may represent species stabilized by nonreducible cross-links (Engel and Furthmayr, 1987). Both RA-5-1 and wild-type F9 aggregates grown without RA (Fig. 11, lanes *E* and *G*) synthesized collagen IV protein, while none could be detected in either cell line grown in monolayer culture without RA (Fig. 11, lanes *A* and *C*).
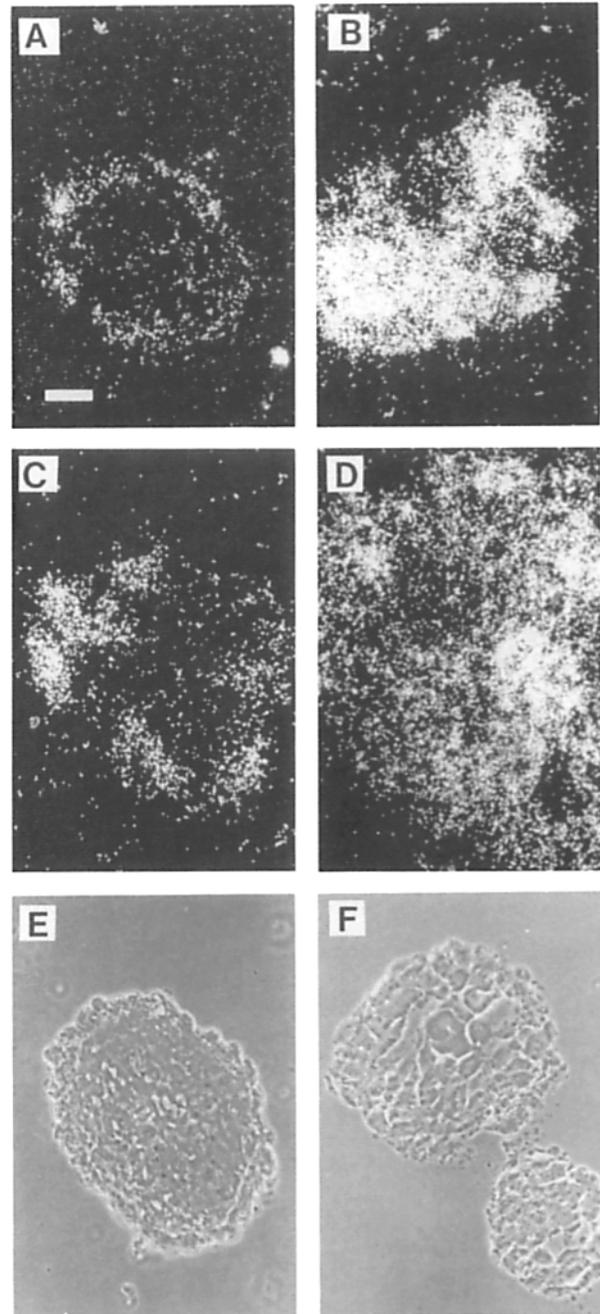

Figure 10. In situ hybridization of ^{35}S-labeled antisense collagen IV(α1) probe to RA-5-1 and wild-type day 8 F9 aggregates. Dark-field micrographs of hybridized sections of RA-treated F9 (*A*) and RA-5-1 (*B*) aggregates, and untreated F9 (*C*) and RA-5-1 (*D*) aggregates are shown. Since large numbers of silver grains in regions of strong signal decrease the clarity of phase micrographs the corresponding phase micrographs are not shown; however, phase microscopy indicated that, as shown in Fig. 7 and 8, the absence of silver grains in the center of the F9 aggregates was not because of an absence of cells in the center. In view of the difficulty in obtaining good phase pictures of sections obscured by numerous silver grains, phase micrographs of nonhybridized, RA-treated F9 (*E*) and RA-5-1 (*F*) aggregates are shown to illustrate the morphological differences between these lines. Exposure time, 4 d. Bar, 25 μm.
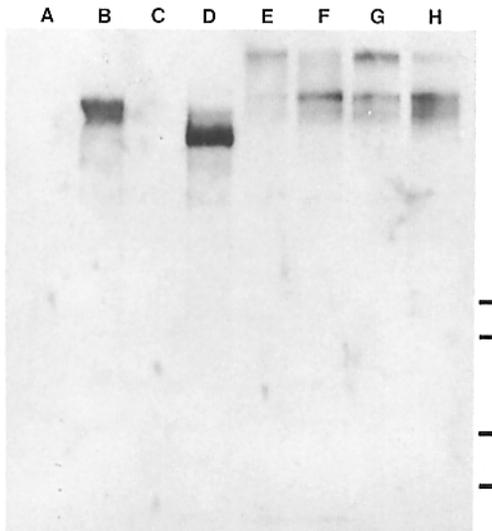
Figure 11. Western analysis of collagen IV protein in RA-5-1 and wild-type F9 cells. Total cellular protein was solubilized, electro-phoresed, and blotted to nitrocellulose. The blot was blocked then incubated in rabbit anti–mouse collagen IV antibody followed by [^{125}I]protein A. (Lanes *A* and *B*) F9 cells grown in monolayer; (lanes *E* and *F*) F9 d8 aggregates; (lanes *C* and *D*) RA-5-1 cells grown in monolayer; (lanes *G* and *H*) RA-5-1 aggregates. (Lanes *A, C, E,* and *G*) no RA; (lanes *B* and *D*) 5×10^{-7} M RA, 2.5×10^{-4} M dibutyryl cAMP, and 5×10^{-4} M theophylline; (lanes *F* and *H*) 5×10^{-8} M RA. 12 μg of total protein was loaded in lanes *A–D*, and 30 μg in lanes *E–H*. The bars indicate the migration of the 110-, 94-, 84-, and 67-kD molecular mass markers.

Immunocytochemical analyses were performed to determine the pattern of collagen IV protein deposition in the RA-5-1 aggregates. Incubation of sections of aggregates grown 8 d in the presence of RA consistently revealed the presence of an organized ECM under the outer layer of cells in wild-type F9 aggregates (Fig. 12, *A* and *B*). The fluorescence observed in RA-5-1 aggregates, however, was highly disorganized and never was found in a contiguous ECM (Fig. 12, *E* and *F*). Both RA-5-1 and wild-type F9 aggregates grown in the absence of RA showed less fluorescence under the outer cell layers and an increased level of disorganized interior fluorescence (Fig. 12, *C, D, G,* and *H*).

Discussion

The formation of mature, VE-containing embryoid bodies from F9 embryonal carcinoma cells is a multistep process. An early RA-associated event is an increase in the abundance of the ERA-1/Hox 1.6 message within 6 h of RA addition (Figs. 1 and 2). The laminin B1 and B2 and collagen IV(α1) messages increase by 4 d after adding RA to aggregates and AFP mRNA is detectable by day 8 (Fig. 1). This data suggests that the expression of five genes regulated during the formation of VE from F9 wild-type stem cells occurs in an ordered, stepwise fashion with the homeobox-containing gene, ERA-1/Hox 1.6, expressed first, followed by the ECM genes laminin B1 and B2 and collagen IV(α1), and lastly by AFP and H19.

The distribution of the laminin B1 and B2, collagen IV, and AFP proteins in F9 aggregates has been described. For

example, several researchers have shown that in the absence of RA or the presence of excess laminin, laminin protein is dispersed throughout sections of day 7 and 8 aggregates. In contrast, the addition of RA causes laminin deposition under the outer layer of cells, with lower amounts in the interior (Grover et al., 1983*a, b*; Grabel and Casanova, 1986). In the in situ hybridization experiments reported here we found that the distribution of the laminin B1 and B2 messages in RA treated aggregates generally reflects that of the proteins (Figs. 5 and 6).

The distribution of the laminin B1 and B2, collagen IV, puzzling. We and others have demonstrated that antibodies to collagen IV uniformly stain aggregates grown without RA, while the addition of RA causes collagen IV to accumulate under the outer cell layer (Grover et al., 1983*b*; Fig. 12). The collagen IV(α1) message, however, is found exclusively in the outer cell layer independent of the presence of RA (Figs. 7 and 8). This result suggests that while RA treatment increases the overall collagen IV mRNA level (Figs. 1 and 2) and alters the pattern of protein deposition, RA does not significantly alter the collagen IV(α1) mRNA distribution.

In RA-treated F9 embryoid bodies, AFP protein is located primarily in the outer cell layer although some can be detected in the inner cells (Grover et al., 1983*a*; Grover and Adamson, 1986; Grabel and Casanova, 1986). We found that the AFP message was synthesized by only a few of the outer cells (Fig. 4). It is possible that the VE layer of F9 embryoid bodies is only partially differentiated or that two different cell types are induced, of which only one produces large amounts of AFP mRNA.

When we examined the capacity of a mutant F9 cell line, RA-5-1, to form VE, we found that RA-treated RA-5-1 aggregates exhibit a sixfold lower AFP mRNA level than wild-type F9 embryoid bodies (Fig. 3). In addition, the message level for another VE marker, H19, is fourfold lower. Thus, the RA-5-1 mutant is clearly impaired in its ability to form VE in response to RA. Further comparison of gene expression in wild-type and mutant RA-5-1 cells determined that the ERA-1/Hox 1.6 mRNA is as rapidly induced in RA-5-1 cells as wild-type F9 cells (Fig. 2). Hence, the first known molecular response to RA by the mutant cells is apparently normal.

Laminin B1 mRNA abundance in the RA-5-1 cell line resembles that of the wild type cells (Fig. 3), but a striking 6–20-fold increase in collagen IV(α1) mRNA abundance is observed in the RA-5-1 aggregates relative to wild-type F9 aggregates (Fig. 2). In situ hybridization of collagen IV(α1) probe to RA-5-1 aggregates grown 8 d in the presence or absence of RA confirmed the overexpression of this gene (Fig. 10). Moreover, this mRNA is distributed evenly in all cells throughout the aggregate rather than primarily in the outer layer of cells.

When collagen IV protein was analyzed on Western blots, the previous observation that RA-treated RA-5-1 cells grown in monolayer secrete lower molecular mass collagen IV than wild-type F9 cells was confirmed (Fig. 11). Surprisingly, the molecular mass and abundance of collagen IV in wild-type F9 and RA-5-1 aggregates are comparable (Fig. 11). This finding raises two questions: (*a*) Why is the apparent molecular mass of collagen IV identical in RA-5-1 and wild-type F9 aggregates, but not in cells cultured in monolayer? And (*b*) why, despite collagen IV(α1) mRNA levels 6–20-fold
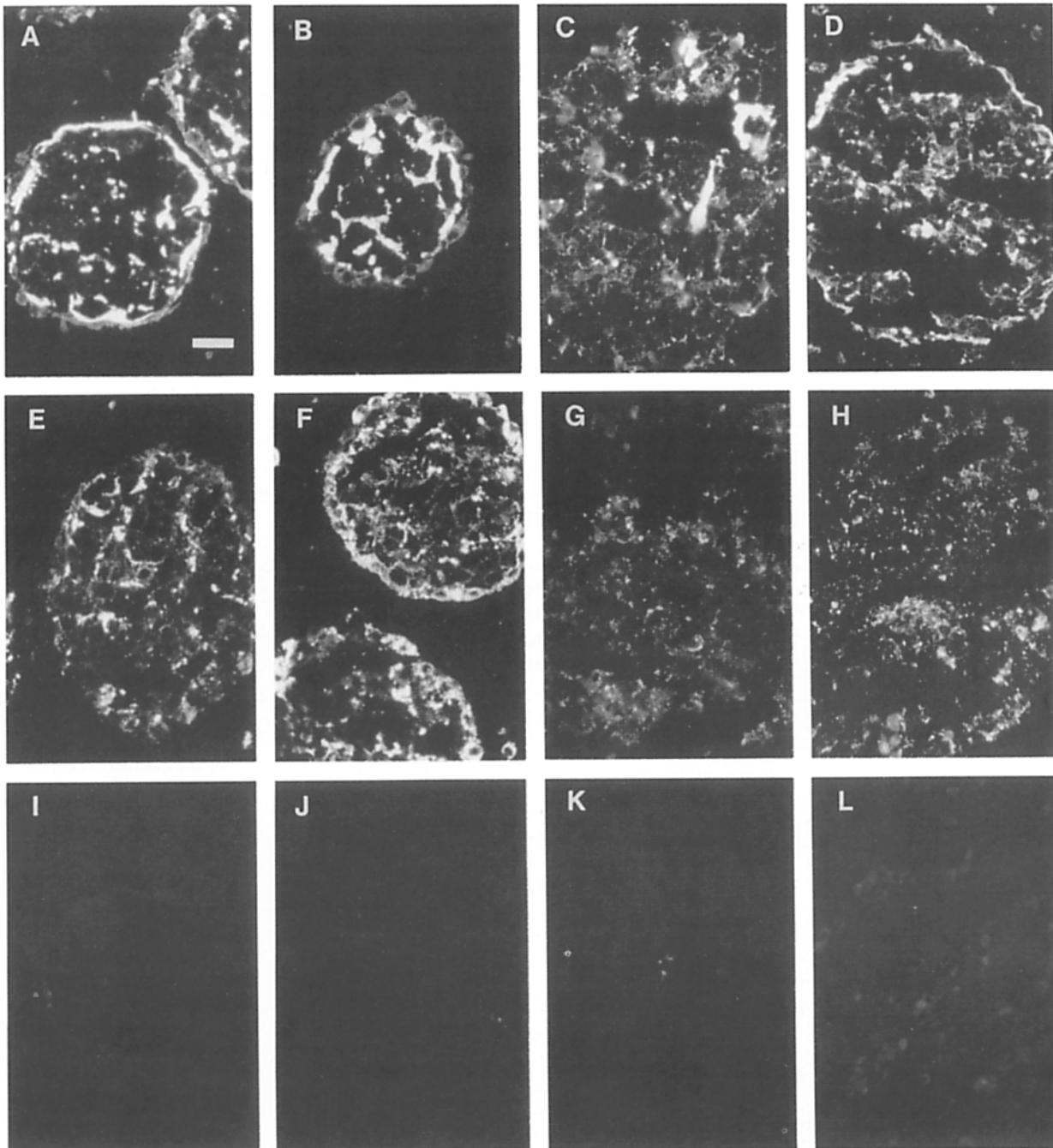
Figure 12. Immunocytological localization of collagen IV protein in sections of RA-5-1 and wild-type F9 aggregates grown 8 d. *A, B, E,* and *F* show sections of RA-treated F9 (*A* and *B*) and RA-5-1 (*E* and *F*) aggregates. *C, D, G,* and *H* show sections of untreated F9 (*C* and *D*) and RA-5-1 (*G* and *H*) aggregates. The sections shown were incubated with rabbit anti–human collagen IV antibody (*A, C, E,* and *G*) or rabbit anti–mouse collagen (*B, D, F,* and *H*) followed by rhodamine-conjugated goat anti–rabbit IgG antibody. *I–L* show sections incubated with normal rabbit serum followed by rhodamine-conjugated goat anti–rabbit IgG; RA-treated F9 aggregates (*I*); RA-treated RA-5-1 aggregates (*J*), untreated F9 aggregates (*K*), and untreated RA-5-1 aggregates (*L*). The photographic exposure time was 8 s for *A, C, E, G,* and *I–L,* and 4 s for *B, D, F,* and *H.*

higher than in wild-type F9 cells, do collagen IV protein levels in mutant aggregates resemble those in wild-type aggregates? Part of the answer may be that the Western blot analysis of cells grown in monolayer detects exclusively intracellular collagen IV, while that of aggregates detects both intracellular collagen IV and extracellular collagen IV trapped between the aggregated cells. Moreover, Western blot analyses measure total protein levels, not rates of protein synthesis. Thus, if collagen IV in RA-5-1 aggregates is unstable, the observable collagen IV would represent only a fraction of the total collagen IV synthesized. If they were possible, pulse-chase experiments employing radioactive amino acids would determine whether collagen IV synthesis rates are changed in mutant aggregates, but these are difficult

to perform because one cannot ensure that every cell in the aggregate is labeled. These arguments do not entirely explain the differences observed between cells grown as monolayers and aggregates, because Wang et al. (1989) showed that underhydroxylated collagen IV can be secreted. Since protein processing often varies from tissue to tissue (Kornfeld and Kornfeld, 1985) collagen IV processing may differ between monolayer and aggregated cells. Because the RA-5-1 prolyl-4-hydroxylase is active, albeit with a threefold higher Km (Wang et al., 1989), these differences might permit additional hydroxylation in aggregated RA-5-1 cells.

Immunohistochemical localization of the collagen IV protein showed that although collagen IV is secreted in the RA-5-1 aggregates after RA treatment, the thick organized basement membrane seen in wild-type cells is not present (Fig. 12). Thus, although the collagen IV protein in RA-5-1 aggregates appears similar in molecular mass to that in wild-type F9 embryoid bodies and secretion of the protein occurs, the pattern of deposition is altered. We do not know if the RA-5-1 prolyl-4-hydroxylase mutation is in the α or β subunit, but it is interesting that the β subunit of prolyl-4-isomerase catalyzes protein disulfide bond formation (Tasanen et al., 1988). It may be that although hydroxylation occurs, collagen IV disulfide bond formation is disrupted, thus changing collagen IV conformation and affecting both its interactions with other basement membrane components and its stability.

These results are consistent with the hypothesis that complete differentiation of F9 cells into VE is a stepwise process requiring the expression of required genes in an ordered fashion. Furthermore, normal basement membrane synthesis may be necessary for the normal expression of some genes in this pathway; specifically collagen IV(α1), and the final differentiation products AFP and H19.

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