Selection and Characterization of F9 Teratocarcinoma Stem Cell Mutants with Altered Responses to Retinoic Acid*

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Retinoic acid induces the differentiation of many murine teratocarcinoma stem cell lines. To elucidate the molecular mechanism of action of retinoic acid, we have selected a series of mutants which exhibit altered differentiation responses to retinoic acid. All of the mutants display abnormal morphology following addition of $5 \times 10^{-7}$ M retinoic acid (RA) and dibutylryl cAMP. In addition, none of the mutants are resistant to the cytotoxic effects of higher concentrations of retinoic acid (greater than 75 nM). After the addition of retinoic acid, one mutant, RA-3-10, does not differentiate by any of the biochemical criteria we have used; this mutant also possesses less than 5% of the wild type level of cellular retinoic acid binding protein (CRABP). Other mutants, such as RA-3-3, RA-3-4, and RA-5-1, contain the same amount of CRABP as wild type F9 cells. However, the mutants RA-3-3 and RA-3-4 exhibit lower levels of plasminogen activator activity, and RA-3-4 also exhibits only 10-20% of the wild type synthesis and secretion of laminin and collagen IV following treatment with RA. After RA treatment of the mutant RA-5-1, laminin and collagen IV are synthesized and secreted at reduced rates relative to wild type cells, and the secreted collagen IV has a lower molecular weight than that of wild type; this suggests that RA-5-1 cells have a mutation in one of the enzymes responsible for post-translational modification of collagen IV. None of the mutants tested exhibits alterations in either cytosolic or membrane bound cAMP-dependent protein kinase activity. These studies provide genetic evidence that the CRABP is required for the differentiation of F9 teratocarcinoma stem cells by retinoic acid. However, even in the presence of CRABP, other types of alterations, such as synthesis of collagen IV with an abnormal molecular weight, appear to cause alterations in the differentiation response of cells to retinoic acid.

Mouse teratocarcinomas contain malignant stem cells which resemble early embryonic cells in some respects, suggesting that these teratocarcinoma stem cells can be used as a model system for early mammalian development. Stem cells from a variety of different lines in culture differentiate spontaneously into several cell types under conditions such as culture at high cell density, and formation of multicellular aggregates (1, 2). Stem cells from many cultured teratocarcinoma lines are also capable of differentiating in response to low concentrations of retinoic acid, a derivative of vitamin A (retinol) (3, 4, 5).

F9 teratocarcinoma stem cells, which differentiate spontaneously at a very low frequency, differentiate in monolayer culture into a homogeneous population of primitive endoderm cells in response to retinoic acid, and into a population of peribital endoderm cells in response to retinoic acid and dibutylryl cyclic AMP (6). This differentiation response of F9 cells is irreversible, fairly synchronous, and reasonably rapid (within 24-48 h) (6). Dibutylryl cyclic AMP treatment results in an enhancement of the retinoic acid induction of F9 stem cell differentiation; dibutylryl cyclic AMP alone has no effect on the cells (6). In contrast, if F9 cells are treated with retinoic acid when they are in small aggregates, cells on the outer surface differentiate into visceral endoderm (7).

Other teratocarcinoma stem cell lines differentiate into different cell types in the presence of retinoic acid. For example, P19 teratocarcinoma stem cell aggregates develop into neuronal and glial cells in the presence of retinoic acid (5).

In addition to teratocarcinoma cells, vitamin A and its derivatives (retinoids) can influence differentiation in several types of cells in culture, including keratinocytes (8), human promyelocytic leukemia cells (9), and melanoma cells (10, 11). The molecular mechanism(s) by which retinoids regulate differentiation of these various cell types is unclear at the present time. Retinoids have been shown to influence specific keratin mRNA content (transcription, processing, or stability of mRNA) in cultured human keratinocytes (8). Retinoids also exert effects on the post-translational glycosylation of proteins (12, 13), and on the growth kinetics of several types of cells in culture (14-16).

There is some evidence that retinoids may act in a manner which is analogous to that of steroid hormones. Specific cellular binding proteins for retinol (CRBP)1 and retinoic acid (CRABP) exist in several tissues (17-19). It has been suggested that these binding proteins mediate the actions of retinoids (20). In the mouse teratocarcinoma stem cell line PCC4.aurR, a cytoplasmic retinoic acid binding protein is present with a sedimentation coefficient of approximately 2 S by sucrose gradient analysis (21, 22). Nuclear translocation of a specific [3H]retinoic acid-CRABP complex has been demonstrated in Y-79 retinoblastoma cells (23), and in

1 The abbreviations used are: CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; PBS, phosphate-buffered saline; HMBA, hexamethylenesulamide; NaDodSO₄, sodium dodecyl sulfate; RA, retinoic acid; Mes, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminomethyl ether) N,N,N',N'-tetraacetic acid.
Tetradecinoma Retinoic Acid Differentiation Mutants

Preparation of Samples for Electrophoresis—The [3S]methionine labeled cells in the wells were washed twice with cold PBS, and then 30 μl of final sample buffer (2% NaDodSO₄, 6% glycerol, 0.126 M Tris-HCl, pH 6.8, 2.5% β-mercaptoethanol, and 0.1% bromophenol blue) was added to each well containing cells for one-dimensional electrophoresis. The samples were boiled for 2 min, and stored at −20 °C until gel analysis.

The medium containing [3S]labeled secreted proteins was precipitated with 10% trichloroacetic acid and the precipitated proteins were washed twice with cold ether to remove the trichloroacetic acid, and resuspended in final sample buffer (see above). Within one experiment, either the same number of trichloroacetic acid-precipitable counts of [3S]methionine-labeled protein was added to each lane of a gel, or the volumes loaded were normalized to the intracellular [3S]methionine-labeled protein counts, as indicated.

Gel Electrophoresis—One-dimensional polyacrylamide gel electrophoresis was performed according to Laemmli (28).

Immunoprecipitation of Proteins—The appropriate antiserum was added to 100–200 μl of [3S]methionine-labeled conditioned medium. The intracellular proteins were dissolved in PBS plus 1% Triton X-100 and phenylmethylsulfonyl fluoride; the proteins were centrifuged at 10,000 × g for 5 min and the supernatant was used for immunoprecipitation. After 1.5 h at 4 °C, 10 μl of protein A-agarose (Sigma) was added and the sample was mixed continuously at 4 °C for 1.5 h. The beads were washed three times with centrifugation in PBS plus 1% Triton X-100, then once in 0.01 M Tris, pH 6.8, and finally boiled for 2 min in 30 μl of final sample buffer before electrophoresis.

Plasminogen Activator Assay—Plasminogen-dependent fibrinogen degradation was measured according to the procedure of Unkeless et al. (29). Conditioned medium from the F9 cells, grown in Costar 24-well plates, was removed from wells and assayed 72 h after retinoic acid addition. It was necessary to normalize the plasminogen activator activity to the number of cells at 72 h after retinoic acid addition, since some of the mutants (i.e. RA-3-10) continued to proliferate rapidly after retinoic acid addition, whereas the growth rate of F9 wild type cells was reduced.

Retinoic Acid Binding Studies—Retinoic acid binding analyses were performed according to established procedures (17, 21, 25). Approximately 150 μl of each of the cytosol fractions (600 μg of protein) were incubated for 3–4 h at 4 °C in the dark with all-trans-[3H]RA (75 nM, 28.7 Ci/mmol) in the absence or presence of 100-fold molar excess of unlabeled RA. Unbound retinoic acid was added, and the samples were washed twice with cold 5% trichloroacetic acid. A partially purified protein inhibitor of the CAMP-dependent protein kinase and the CAMP-independent protein kinase was used to distinguish between the activities.

UV-absorbing protein was precipitated by 20% trichloroacetic acid on ice; the precipitated proteins were washed twice with cold ether to remove the trichloroacetic acid, and resuspended in a small volume of methanol. Purified [3H]retinoic acid was concentrated to 5,000 cpm in a small volume of methanol.

The absorbance at 205 nm of final sample buffer (2% NaDodSO₄, 6% glycerol, 0.126 M Tris-HCl, pH 6.8, 2.5% β-mercaptoethanol, and 0.1% bromophenol blue) was used to determine the amount of protein in each sample. The absorbance at 300 nm was also used to estimate the amount of protein in each sample.

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The medium containing [3S]labeled secreted proteins was precipitated with 10% trichloroacetic acid and the precipitated proteins were washed twice with cold 5% trichloroacetic acid. A partially purified protein inhibitor from bovine brain (33) was used to distinguish between the activities of the CAMP-dependent protein kinase and the CAMP-independent protein kinases.
RESULTS

Selection of F9 Mutants with Altered Differentiation in Response to Retinoic Acid—The selection procedure is based on our observation that wild type F9 stem cells form colonies in semisolid agarose, but wild type F9 stem cells treated with 1 μM retinoic acid differentiate and do not form colonies. Therefore, mutants which exhibit either no differentiation response, or only a slight response to retinoic acid should form colonies when plated in semisolid agarose in the presence of retinoic acid.

We plated approximately 1 x 10^7 F9 stem cells/100-mm tissue culture dish in semisolid agarose in the presence of 1, 2, 5, 10, 20, or 50 μM retinoic acid. The cloning efficiencies in the absence of the drug ranged from 0.5 to 4% in semisolid agarose. From a mutagen-treated population of F9 cells, we obtained mutants which formed colonies in the presence of 5, 10, 20, and 50 μM retinoic acid at frequencies of 0.5–2 x 10^-5. The frequency of obtaining spontaneous mutants was less than 1 x 10^-7. These mutants were picked from the semisolid agarose, expanded in the absence of retinoic acid, frozen in liquid nitrogen, and characterized as described in the following sections. The mutants RA 3-3, RA 3-4, and RA-3-10 were obtained from one selection; the mutant RA-5-1 was obtained in another selection attempt.

Growth of the Mutants in Liquid Culture—The mutants exhibit growth rates in the absence of retinoic acid which are fairly similar to the growth rate of F9 wild type stem cells (Fig. 1). Only one of the mutants, RA-3-4, grows more slowly than wild type F9 stem cells. The mutant RA-3-4 exhibits a generation time in the absence of retinoic acid of 20–22 h, whereas the generation time of wild type cells is 16–18 h.

In the presence of relatively low nontoxic concentrations of retinoic acid (0.5–50 μM), the growth rate of F9 wild type cells decreases as the cells differentiate into primitive endoderm cells (Fig. 2). One of the mutants, RA-3-3, also exhibits this decrease in growth rate, but three other mutants (RA-3-4, RA-3-10, and RA-5-1) decrease their growth rate only slightly in the presence of 0.5–50 μM retinoic acid (Fig. 2). This decrease in growth rate in the presence of low concentrations of retinoic acid is correlated with the degree of differentiation in the mutants as measured morphologically and biochemically (see below).

At higher concentrations (greater than 75 μM), retinoic acid exerts toxic effects on the F9 wild type stem cells, and on the various mutants (Fig. 2). Stem cells detach from the tissue culture dish within 24–48 h following the addition of high concentrations of retinoic acid, so we have not been able to ascertain whether the stem cells begin to differentiate before they are killed in the presence of these high toxic retinoic acid concentrations. The wild type F9 stem cells and the various mutant stem cell lines appear to be equally sensitive to the toxic effects of high retinoic acid concentrations. This result suggests that the cytotoxic effects of high retinoic acid concentrations are mediated by a different mechanism from the effects of retinoic acid on stem cell differentiation.

Morphology of the Mutants—The morphology of wild type F9 stem cells is shown in Fig. 3a; the cells grow in culture as closely packed colonies, and it is often difficult to distinguish cell-cell boundaries. Following the addition of 1 μM retinoic acid plus 1 mM dibutyryl cyclic AMP and 590 μM theophylline to F9 wild type cells, the cells become more rounded and refractile, with processes which extend many cell diameters (Ref. 6; and Fig. 3b).

Although the mutant stem cell lines have morphological characteristics which are similar to wild type F9 stem cells in the absence of retinoic acid (data not shown), the various mutant lines exhibit altered morphology following the addition of retinoic acid and dibutyryl cyclic AMP. The mutants RA-3-3, RA-3-4, and RA-5-1 partially differentiate upon treatment of the cells with retinoic acid and dibutyryl cyclic AMP; they extend short processes but they do not become as round and refractile as wild type F9 cells (Fig. 3f). The mutant RA-3-10 does not exhibit the characteristic morphology of differentiated wild type F9 cells (Fig. 3f); in fact, even after treatment with retinoic acid and dibutyryl cyclic AMP for 5 days, the RA-3-10 mutant cells appear indistinguishable from F9 wild type untreated stem cells (Fig. 3f).

Induction of Specific Proteins by Retinoic Acid—Plasminogen activator production is elevated in F9 cells following treatment with retinoic acid; the production of plasminogen activator is further enhanced by treatment with dibutyryl cyclic AMP (6). Dibutyryl cyclic AMP addition alone (without retinoic acid) caused no elevation of plasminogen activator production (6). We measured the levels of plasminogen activator secreted into the medium from wild type F9 and mutant

![Figure 1](http://www.jbc.org/)  
*Fig. 1. Growth curves of F9 wild type stem cells (○), RA-3-3 (△), RA-3-4 (●), and RA-3-10 (□) lines in the absence of retinoic acid. The cells were seeded at the densities shown in Costar 12 FM-multiples plates. At intervals thereafter, cells were trypsin treated in duplicate cultures and counted using a Coulter counter. The differences between the number of cells in the duplicates was less than 10%.

![Figure 2](http://www.jbc.org/)  
*Fig. 2. Effect of retinoic acid on the growth of F9 wild type and mutant lines in liquid culture. This experiment was performed as described under "Experimental Procedures." Growth over a 72-h period in the presence of retinoic acid is expressed as a percentage of control growth, i.e. growth in the absence of etoxogens retinoic acid. The symbols are: ○, wild type F9; △, RA-3-3; ●, RA-3-4; □, RA-3-10; and ▲, RA-5-1. This experiment was performed twice with similar results.
cells, both before and after treatment with retinoic acid and dibutyryl cyclic AMP. The results indicate that the mutants RA-3-3, RA-3-4, and RA-5-1 exhibited some increase in plasminogen activator activity following the addition of retinoic acid alone, or retinoic acid plus dibutyryl cyclic AMP (Fig. 4). However, none of the mutants exhibited increases in plasminogen activator activity which were as large as the increase for wild type F9 cells following retinoic acid addition (Fig. 4). The mutant RA-3-10 did not display any increase in plasminogen activator activity after retinoic acid addition (Fig. 4).

We then measured the synthesis and secretion of laminin and collagen IV in the mutants after treatment with retinoic acid, or retinoic acid plus dibutyryl cyclic AMP, since secretion of these extracellular matrix proteins constitutes a biochemical marker for differentiated parietal endoderm cells (3, 6). The synthesis and secretion of laminin and collagen IV in the mutant RA-3-3 appeared to be similar to that of wild type F9 cells (Fig. 5, lanes a–c, versus lanes j–l). The mutant RA-3-4 synthesized and secreted some laminin and collagen IV, but the levels of these proteins in the mutant RA-3-4 were only 10–20% of wild type levels (Fig. 5, lanes d–f), as determined by densitometry of the gel shown in Fig. 5. The mutant RA-3-10 did not secrete any detectable laminin, and only 2% of wild type F9 levels of collagen IV following retinoic acid addition (Fig. 5, lanes g–i). Gel electrophoreses of the intracellular proteins, immunoprecipitated with laminin, or collagen IV antisera, from the mutant RA-3-10 following retinoic acid addition demonstrated that no detectable laminin or collagen IV was synthesized (data not shown). This result suggests that the mutant RA-3-10 does not have a defect related to protein secretion, but rather that the mutant RA-3-10 does not respond to retinoic acid.

The mutant RA-5-1 exhibited a very intriguing response to the addition of retinoic acid (Fig. 6A). RA-5-1 cells secreted only about 10% of the laminin that differentiated F9 wild type cells secreted (Fig. 6A, lane a versus b). The mutant line RA-5-1 also secreted collagen IV, but this collagen IV had an altered molecular weight (Fig. 6, A, lane b; B, lane b). When the intracellular proteins in retinoic acid-treated wild type and RA-5-1 cells were examined by immunoprecipitation with anti-laminin or anti-collagen IV serum, it was evident that the RA-5-1 cells synthesized less laminin than wild type cells (Fig. 6A, lane c versus h). It was also clear that the RA-5-1 cells synthesized collagen IV of a lower molecular weight than that in wild type F9 cells (Fig. 6A, lane f versus i). In addition, the collagen IV from the mutant RA-5-1 immunoprecipitates as a smear rather than as two distinct bands. This can be

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Changes in morphology of F9 wild type cells and mutant lines in response to retinoic acid plus dibutyryl cyclic AMP. Wild type F9 undifferentiated stem cells, a; F9 wild type, 72 h after addition of 1 μM retinoic acid, 1 mM dibutyryl cyclic AMP, and 500 μM theophylline, b; RA-3-3 cells, treated as in b with drugs, c; RA-3-4 cells, treated as in b with drugs, d; RA-5-1 cells, treated as in b with drugs, e; RA-3-10 cells, treated as in b with drugs, f. All figures are phase contrast micrographs; magnification, × 125.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Plasminogen activator activity of F9 wild type and mutant cell lines 72 h after the addition of retinoic acid (1 μM), or retinoic acid (1 μM) plus dibutyryl cyclic AMP (1 mM) and theophylline (500 μM). The plasminogen activator assay was performed as described under "Experimental Procedures." The plasminogen activator activity is expressed as 125I-fibrin counts/min released per 10⁶ cells. These counts were between 5 and 50% of the total 125I-fibrin counts/min released upon trypsin-treatment of the dishes. The results were obtained by assaying conditioned medium 72 h after drug addition. F9 wild type (A); RA-3-3 cells (B); RA-3-4 cells (C); and RA-3-10 cells (D). The bars are as follows: undifferentiated stem cells (○), treated 72 h with 1 μM retinoic acid (●), and treated 72 h with 1 μM retinoic acid plus 1 mM dibutyryl cyclic AMP and 500 μM theophylline (□).
better seen in Fig. 6B. For comparison, proteins from a line of mouse parietal endoderm cells, PYS-2, are also shown in Fig. 6A (lanes c, j, and k). Thus, in RA-5-1 mutant cells treated with retinoic acid, less laminin is synthesized and secreted, and the collagen IV that is secreted has an abnormal molecular weight.

We examined the induction of other proteins in wild type F9 cells, and in the mutant lines, following the addition of retinoic acid, or retinoic acid plus dibutyryl cyclic AMP. Using the technique of one-dimensional NaDodSO₄-gel electrophoresis, we observed that three other proteins (99,000, 78,000, and 45,000 daltons) were synthesized at increased rates upon treatment with retinoic acid, less laminin is synthesized and secreted, and the collagen IV that is secreted has an abnormal molecular weight.

**Fig. 5. Autoradiogram of a 5% NaDodSO₄-polyacrylamide gel of [³⁵S]methionine-labeled secreted proteins.** Upper two arrows at right indicate the position of secreted laminin A and B chains, lower two arrows indicate the position of the two secreted chains of collagen IV. Control, untreated cells are in lanes a, d, g, and j. Cells were treated with retinoic acid (1 μM) (lanes b, e, h, k), or retinoic acid (1 μM) plus dibutyl cyclic AMP (1 mM) and theophylline (500 μM) (lanes c, f, i, and l) for 72 h prior to the addition of [³⁵S]methionine. The lanes are identified as follows: a-c, RA-3-3 cells; d-f, RA-3-4 cells; g-i, RA-3-10 cells; and j-l, F9 wild type cells. The number of [³⁵S]methionine counts loaded per lane was normalized to trichloroacetic acid-precipitable intracellular [³⁵S]methionine-labeled protein. Exposure was for 5 days.

electrophoresis so that we can more easily measure differences between wild type and mutant cells with respect to induction of specific intracellular proteins by retinoic acid. However, these results of the NaDodSO₄-gel electrophoresis of proteins from F9 wild type cells and from the mutant cell lines (Fig. 7) are consistent with the results we obtained for laminin and collagen IV synthesis; the mutant RA-3-10 exhibited no response to retinoic acid, and the mutants RA-3-3, RA-3-4, and RA-5-1 demonstrated partial responses relative to wild type F9 cells.

**Intracellular Retinoic Acid Binding Protein**—There is experimental evidence which suggests that the action of retinoic acid is mediated by a cellular retinoic binding protein, as discussed in the introduction (24). However, the involvement of an intracellular binding protein in the action of retinoic acid in various cell types remains controversial. We examined F9 wild type stem cells, and the various mutants, for the presence of a functional CRABP. Sucrose gradient analyses of the binding of [³⁵S]retinoic acid to cytosol supernatants of cell homogenates were performed. The 100,000 x g supernatant fraction of F9 wild type cells bound [³⁵S]retinoic acid, displaying a single symmetrical peak with a sedimentation coefficient of approximately 2 S (Fig. 8A). Quantitation of the CRABP in the F9 wild type undifferentiated stem cells gave values of approximately 0.3 pmol/mg of cytosolic protein.

The mutants RA-3-3 (data not shown) and RA-3-4 (Fig. 8B) possessed cellular retinoic acid binding proteins which were similar to the CRABP of wild type F9 cells by this assay. In contrast, the mutant RA-3-10 did not demonstrate binding of [³⁵S]retinoic acid, within the detection limits of this assay (approximately 20 fmol/mg of cytosolic protein) (Fig. 8C). Although in this experiment it appears that RA-3-10 cells possess a very small amount of [³⁵S]retinoic acid binding activity, in two other experiments the RA-3-10 cells displayed no measurable binding of [³⁵S]retinoic acid. The mutant line RA-5-1 exhibited a level of [³⁵S]retinoic acid binding which was similar to the level in F9 wild type stem cells (data not shown).

These sucrose gradient sedimentation assays (Fig. 8) were performed on wild type F9, and mutant undifferentiated stem cell lines, without any retinoic acid added. Using F9 wild type cells, we have measured the level of [³⁵S]retinoic acid binding 72 h after retinoic acid was added to the medium to induce differentiation. The level of [³⁵S]retinoic acid binding in extracts from the retinoic acid-treated wild type F9 cells was similar to the level of binding in the F9 wild type stem cells (data not shown).

**Cyclic AMP-dependent Protein Kinase Activity in the Mutant Cell Lines**—There was evidence in the literature (34) that retinoic acid treatment of F9 stem cells caused an increase in both cytosolic and plasma membrane-associated cyclic AMP-dependent protein kinase activities. Thus, although we had some information concerning the biochemical basis of the mutation in the lines RA-5-1 (abnormal collagen IV secretion) and RA-3-10 (lack of detectable CRABP), the possibility existed that either of the mutants RA-3-3 or RA-3-4 might possess an alteration in cyclic AMP-dependent protein kinase activity.

We measured the levels of both cytosolic and plasma membrane-associated cyclic AMP-dependent protein kinase in undifferentiated F9 wild type stem cells, and in the three mutants RA-3-3, RA-3-4, and RA-3-10. There were no differences in the cyclic AMP-dependent protein kinase activities between F9 wild type stem cells, and the various mutant lines (Fig. 9). Moreover, when we measured the cytosolic and membrane-bound cyclic AMP-dependent protein kinase ac-

2 S.-Y. Wang, unpublished results.
quantitation of RI and RII by photoaffinity labeling with 8-cyclic AMP-dependent protein kinase I (RI) and II (RII) after azido-['"PICAMP (data not shown).

activities in wild type F9 cells after 48 h of treatment with retinoic acid (1 μM), we did not observe any increase in cyclic AMP-dependent protein kinase activity (Fig. 10), in contrast to the results of Plet et al. (34). We also did not observe any increase in the amount of regulatory subunits of the cytosolic cyclic AMP-dependent protein kinase I (R₁) and II (R₂) after retinoic acid addition; these experiments were performed by quantitation of R₁ and R₂ by photoaffinity labeling with 8-azido-["P]cAMP (data not shown).

Our results suggest that an alteration in the activity of either the cytosol or membrane-bound cAMP-dependent protein kinase is not the basis of the mutation in either of the mutant lines RA-3-3 or RA-3-4.

**DISCUSSION**

The initial characterization of these mutants which display abnormal differentiation upon treatment with retinoic acid allows us to draw some conclusions concerning the molecular mechanism of the induction of differentiation by retinoic acid. First, we have genetic evidence that the cellular retinoic acid binding protein appears to be required for the induction of F9 stem cell differentiation by retinoic acid. The mutant RA-3-10 exhibits no detectable differentiation response following the addition of retinoic acid, and this mutant also lacks a detectable CRABP which can bind ["H]retinoic acid.

The RA-3-10 cell line may be similar in some respects to the mutant which lacks detectable CRABP described by Schindler et al. (25) in PCC4.azalR cells. The two mutants were isolated using different selection methods, however. In addition, the mutant described by Schindler and colleagues (25) also does not respond to the drug HMBA (35), whereas the RA-3-10 line responds to HMBA in a fashion identical to that of wild type F9 cells. In our experience, HMBA causes a morphological change in both wild type F9, and RA-3-10 cells, but no detectable laminin, collagen IV, or plasminogen activator activity is induced. This morphological change oc-

\[3\] S.-Y. Wang, R. Levine, L. J. Gudas, unpublished results.
curs at HMBA concentrations which are somewhat cytotoxic (20% cell killing), and the morphological change is reversible upon removal of HMBA. Thus, the effects of HMBA are different from those of retinoic acid, which causes irreversible differentiation of F9 wild type stem cells (3, 6). It is not clear why the mutant described by Schindler et al. (25) does not respond to HMBA (35), while the mutant RA-3-10 line, described in this report, does respond, as measured by a morphological alteration.

The mutant RA-3-10 cell line which lacks a detectable CRABP is not resistant to the cytotoxic growth inhibitory effects of high concentrations of retinoic acid (greater than 75 μM), suggesting that these toxic effects are not mediated by the CRABP. This result is consistent with the results from genetic experiments of Lotan et al. (11), who demonstrated that the retinoic acid-induced growth inhibition in S91-C-2 murine melanoma cells was independent of the CRABP level. We conclude that the cellular retinoic acid binding protein mediates the effects of retinoic acid on teratocarcinoma stem cell differentiation, but that cytotoxicity and presumably growth inhibition result from another mechanism.

The RA-3-10 cells, unlike wild type F9 cells, are not induced to differentiate by high concentrations of retinol (1 mM). No synthesis of laminin or collagen IV is detected in RA-3-10 cells after retinol addition, whereas wild type F9 cells did synthesize laminin and collagen IV after retinol treatment. That a functional CRABP is required for differentiation in the presence of retinol suggests that retinol is being converted to retinoic acid in the medium, or within the cells; retinol and retinoic acid bind to different cellular binding proteins, and there is no competition for binding between the two retinoids (21, 24).

The phenotype of the RA-3-10 line, like that of all of the mutant lines described in this report, is quite stable. We routinely thaw frozen stocks of the mutant lines every 4–6 weeks.
months, however, to ensure that the cultures remain phenotypically uniform.

Using the F9 teratocarcinoma stem cell line, we have selected several other mutants which, like RA-3-10, exhibit no detectable differentiation response to retinoic acid. We are currently assaying these mutants for \(^{3}H\)retinoic acid binding. The absence of a CRABP which binds retinoic acid is probably a common mutant phenotype which we select from the mutagenized F9 wild type line.

The other mutants described in this report, RA-3-3, RA-3-4, and RA-5-1, possess normal \(^{3}H\)retinoic acid binding activity, as measured by the sucrose gradient sedimentation assay. There is some evidence that retinoids may act in a fashion similar to that of steroid hormones (21, 36). If an analogy is made with the mechanism of action of steroids (37), other alterations in the CRABP may occur, and these alterations would not be detected in this sucrose gradient assay. For instance, the CRABP-retinoic acid complex may not move to the nucleus, or the complex may not bind properly to chromatin or DNA in the nucleus.

The mutant line RA-5-1 synthesizes collagen IV with a molecular mass which is approximately 20,000 daltons less than that of F9 wild type (Fig. 6), and that in other published reports (38, 39). The RA-5-1 collagen IV also appears as a smear rather than as two distinct bands (Fig. 6). At the present time, we are not sure whether this mutant has a mutation in the collagen gene, or in an enzyme involved in the post-translational modification of collagen IV, although we are exploring this further. That the RA-5-1 mutant was isolated in a single step as a mutant defective in the induction of differentiation by retinoic acid provides genetic evidence that the synthesis and secretion of collagen IV may play a critical role in the differentiation process induced by retinoic acid. In addition, the synthesis and secretion of laminin in the mutant RA-5-1 are also greatly reduced, suggesting that the synthesis and secretion of collagen IV and laminin are coordinately regulated in differentiating F9 wild type cells.

The mutant RA-3-4 appears to differentiate only partially in response to RA; after RA addition, RA-3-4 cells synthesize and secrete laminin and collagen IV at a level that is only 10–20% of that of wild type F9 cells (Fig. 5). We are currently analyzing the mutants RA-3-4 and RA-5-1 further using cDNA clones specific for laminin, and collagen IV (40), in order to learn more about the molecular biology of the differentiation process.

The mutants RA-3-3, RA-3-4, and RA-3-10 did not display any obvious alterations in either cytosolic or membrane-bound cyclic AMP-dependent protein kinase activity (Fig. 9). We also were unable to demonstrate an increase in the cyclic AMP-dependent protein kinases, R\(_1\) and R\(_2\), following retinoic acid treatment of wild type F9 cells (Fig. 10). We cannot explain why our results differ from those of Plet et al. (34), but at the current time we do not believe that any alteration in cyclic AMP-dependent protein kinase activity is the basis for the mutant phenotypes we observe in the mutant lines RA-3-3, RA-3-4, RA-3-10, or RA-5-1.

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Selection and characterization of F9 teratocarcinoma stem cell mutants with altered responses to retinoic acid.

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