An Analysis of Retinoic Acid-induced Gene Expression and Metabolism in AB1 Embryonic Stem Cells*

(Received for publication, January 30, 1996, and in revised form, March 28, 1996)

Anne C. Chen and Lorraine J. Gudas†
From the Department of Pharmacology, Cornell University Medical College, New York, New York 10021

Mature embryonic stem cells such as the AB1 cell line undergo differentiation in the presence of retinoic acid (RA) into an extraembryonic epithelial cell type. This results in the activation of genes such as Hoxa-1, Hoxb-1, laminin, collagen IV(a1), tissue plasminogen activator, RARβ, and CRABPII. The CRABPI gene is regulated in an unusual fashion; CRABPI message and protein levels are induced at low concentrations of RA, but induction is diminished at higher concentrations. AB1 cells take up RA rapidly from the medium, and the addition of low, exogenous concentrations of RA to the culture medium results in very high intracellular RA concentrations. For example, AB1 stem cells cultured in 5 nM [3H]RA have an internal [3H]RA concentration of 1-2 μM within the first hour. AB1 cells also metabolize [3H]RA to more polar RA derivatives. The half-life of RA in AB1 cells not previously exposed to RA is about 2-2.5 h versus 40-45 min in cells cultured for 2-3 days in 1 μM exogenous RA. Thus, the enzyme(s) which metabolize RA are induced or activated by RA. Furthermore, the local concentration of RA required to elicit some biological responses may be higher than previously thought.

Retinoic acid (RA), a derivative of vitamin A, plays a critical role in many cellular processes, including cellular differentiation (1, 2) and embryogenesis (3, 4). RA can also regulate the growth and differentiation of a large variety of pre-malignant and neoplastic cell types, both in vivo and in culture (2, 5–9). RA and several of its analogs have proven to be efficacious as agents in the treatment of dermatologic disorders (10) and as chemotherapeutic agents in the treatment of a number of human cancers (11–15). However, the usefulness of retinoids such as RA in the treatment of human disease is limited by their potent teratogenicity (16–18) and rapid metabolism in many cell types (12, 19).

The mechanisms by which RA exerts its actions are not fully understood, but several classes of proteins involved in mediating the cellular response to RA have been identified. These cellular mediators include at least two families of nuclear retinoid receptors (RARs and RXRs) and two cellular retinoid acid-binding proteins (CRABPI and CRABPII). The nuclear receptors are members of the steroid hormone receptor superfamily and act as high affinity ligand-dependent transcription factors (20, 21). In the presence of various RA isoforms, RARs and RXRs form heterodimers (21-27) that activate differentiation-specific gene transcription by binding to retinoid acid response elements in the promoter regions of RA-responsive target genes (28–33). The CRABPs are cytoplasmic, low molecular weight proteins which are members of the fatty acid-binding protein superfamily (34). The CRABPI and CRABPII proteins are highly related to one another and bind to all-trans-RA with high, but varying affinities (34–38). The functions of the CRABP proteins are not well understood, but they are thought to play an important role in regulating the metabolism of RA (39–41), and in regulating the intracellular levels of RA in various tissues of the developing embryo. This hypothesis is supported by the fact that CRABPI and CRABPII messages are expressed in specific and distinct temporal and spatial patterns in many tissues of the developing embryo, including those tissues that are sensitive to the teratogenic effects of RA (42–48).

In response to RA, many teratocarcinoma cell lines (such as F9, P19, and NT2) (49–52) and embryonic stem cell lines (such as D3 and CCE) (53–55) differentiate into cell types resembling those found in early stages of the developing embryo. The AB1 murine embryonic stem cell line (56) is a pluripotent stem cell line that has been used extensively by many groups to investigate the effects of homozygous deletions of a variety of developmentally-regulated (activin, inhibin, follistatin, Hox b4) (57–61) and growth-related genes (p53, c-myc) (59, 62) in tissue culture and in mice. Although AB1 cells have been widely used to study the effects of gene knockouts, these cells have not been well characterized previously for their response to RA. It is known, however, that AB1 cells undergo differentiation after treatment with RA. In this paper, we characterize the RA response of the AB1 embryonic stem cell line with regard to RA-inducible gene expression and RA metabolism.

MATERIALS AND METHODS

Cell Culture and Reagents—AB1 murine embryonic stem cells (56) were kindly provided by Dr. Allan Bradley (Baylor College of Medicine, Houston, TX). AB1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 x antibiotic/antimycotic (consisting of 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B), 1 mM sodium pyruvate, 100 μM minimal essential medium non-essential amino acids, 1 μM β-mercaptoethanol, and 10% FBS with 100 μM leukemia inhibitory factor (LIF) at 37 °C in 5% CO2. Embryonic stem cells are routinely cultured in the presence of feeder cells to prevent spontaneous differentiation; however, addition of LIF to the culture medium inhibits spontaneous differentiation (63) and allows AB1 cells to be grown without feeder cells. Addition of RA overcomes the inhibitory effects of LIF and induces differentiation (64). All cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY). Retinoic acid (Sigma) was dissolved in 100% ethanol and stored at 4 °C.
Retinoic Acid-induced Gene Expression in AB1 Cells

Retinoic acid was added to cells 24 h after plating; the final ethanol concentration did not exceed 1% of the total volume. [3H]Al-trans-retinoic acid and [α-32P]dCTP were obtained from DuPont NEN (Boston, MA).

Probes—An 800-base pair EcoRI fragment containing the entire CRABPI cDNA was excised from the pMT-CRABPI plasmid (40) for use as a probe for Northern blots. Similarly, an 876-base pair EcoRI/HindII fragment containing the entire CRABPI cDNA was excised from the pMT-CRABPII plasmid. In addition, cDNA fragments corresponding to the murine Hox b1, laminin B1, collagen IV (α1), Tam 2.5 (tissue plasminogen activator), J6 serpin, Rex1, and actin genes were excised from the following plasmids for use as probes. The pHox b1 (also known as Hox b2.9) plasmid was obtained from Dr. Joseph Grippo (Hoffmann-LaRoche, Nutley, NJ) and contains a 435-base pair partial cDNA specific for the murine Hox b1 gene within the EcoRI/HindIII site of pGem7zf (+). The plasmid contains a partial cDNA specific for the murine laminin B1 gene within the EcoRI site of pUC9 (65). The plasmid 381 plasmid contains a partial cDNA specific for the murine collagen IV (α1) gene within the EcoRI site of pUC9 (65). The Tam 2.5 plasmid was kindly provided by Dr. Sidney Strickland (SU NY Stony Brook, Stony Brook, NY) and contains the full-length murine tissue plasminogen activator cDNA cloned into the EcoRI site of pSCT1 (+). The plasmid contains a partial cDNA specific for the murine serpin gene within the PstI site of pUC9 (68). The pRex1-2.5R plasmid contains a 1.7-kilobase pair fragment of the mouse Rex-1 gene cloned into the EcoRI site of pUC9 (69). The pUC Act1 plasmid contains a partial cDNA specific for the murine β-actin gene within the PstI site of pUC9 (70).

RNA Isolation and Northern Blot Analysis—Total cellular RNA was isolated by the guanidinium isothiocyanate method of Chirgwin et al. (71). Cells were plated in medium containing 105 units/ml LIF and treated with varying concentrations of RA 24 h after plating. For the half-life experiment, AB1 cells were cultured in the presence or absence of RA for 72 h, followed by addition of 2 μg/ml actinomycin D (Sigma) for varying time periods. The mRNA was size fractionated on a 1% agarose, 2.2 M formaldehyde gels, transferred to nitrocellulose, and hybridized to random primer-labeled probe (72). Blots were washed once in 0.1% SSC, 0.1% SDS for 20 min at room temperature, once in 2× SSC, 0.1% SDS at 60°C, and once in 0.2× SSC, 0.1% SDS at 60°C. Autoradiographs were quantitated by scanning densitometry using an LKB densitometer (Pharmacia LKB, Piscataway, NJ). All of the Northern experiments described in this paper were performed at least twice from independent RNA preparations.

Preparation of Cytoplasmic Protein Extracts and Polyacrylamide Gel Electrophoresis—Cytoplasmic protein extracts were prepared as described (40). Briefly, cells were resuspended in 10 mM Tris, pH 7.4, and 7 mM β-mercaptoethanol and solubilized by Dounce homogenization. Supernatants were obtained by ultracentrifugation at 105,000 g for 1 h at 4°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad) as per the manufacturer’s instructions. Bovine serum albumin (Sigma) was used as a standard. Protein extract was incubated with 50 mM [3H]all-trans-retinoic acid (DuPont NEN) and 2 mM diethiothreitol for 16 h at 4°C in the presence or absence of a 500-fold excess of unlabelled retinoic acid. The samples were then analyzed by the non-denaturing polyacrylamide gel electrophoresis (PAGE) technique described in Siegenthaler and Saurat (73) and Siegenthaler et al. (74), except that samples were resolved on a 12% polyacrylamide gel and 0.32× Tris, pH 8.4, was used in the separating gel. The sample containing lanes of the gel were sliced into 2-mm bands. Each gel slice was incubated overnight at 50°C in SOLVABLE tissue solubilizer (DuPont NEN) and tritiated counts were quantitated by liquid scintillation counting in ATOMLIGHT scintillant (DuPont NEN).

Electrophoresis—Electrophoresis of cytoplasmic protein extracts was performed using a gradient from 0% acetonitrile (15 mM ammonium acetate, pH 6.5) to 100% acetonitrile (75). Non-labeled retinoid standards were run concurrently and were monitored at a wavelength of 340 nm while a Packard A-500 radiochromatography detector (Packard Instruments, Downers Grove, IL) was employed to monitor the labeled retinoids.

RESULTS

AB1 Cells Express Retinoic Acid-inducible Genes—AB1 cells were cultured in the presence of LIF, either without (AB1 stem cell control), or with 1 μM RA for various lengths of time. Upon treatment with RA, AB1 cells differentiate into cells resembling extraembryonic endoderm. AB1 cells also behave similarly to other embryonic stem cell lines and teratocarcinoma cell lines with respect to the expression of many RA-responsive genes (Fig. 1). Some early-response genes such as the homeobox-containing genes, Hox b1 (Fig. 1) (76, 77) and Hox a1 (data not shown) (78), are induced by RA, while the zinc-finger containing Rex1 gene (55, 69) is down-regulated by RA (Fig. 1). In addition, late-response genes such as collagen IV (α1) (65), tissue plasminogen activator (Tam 2.5) (66, 67), laminin B1 (65), and J6 serpin (68, 79) are induced after RA treatment (Fig. 1). The RARβ gene is also induced in response to RA (data not shown). All of these genes are expressed to similar degrees and with similar kinetics as compared to what has been observed previously in F9 teratocarcinoma cells (6, 65, 67–69, 78, 80). These results demonstrate that AB1 cells respond to RA by differentiating along an epithelial pathway to parietal endoderm (2) and by inducing or down-regulating a large number of genes.

Expression of CRABPI and CRABPII Genes—The genes encoding the cellular retinoic acid-binding proteins, CRABPI and CRABPII, are also regulated by RA (34). While CRABPI mRNA expression is induced after RA treatment of both F9 and
P19 cells (36), CRABPI message is reduced by RA in F9 cells (35), but induced by RA in P19 cells (50). The mechanism for the differential regulation of the CRABPI gene in F9 and P19 cells is not known. While less is known about the regulation of the CRABPI gene (35, 81, 82), the transcriptional regulation of the CRABPII gene has been reported to occur via high affinity RA response elements (32, 33).

AB1 cells were cultured in LIF in the presence of a range of RA doses for varying lengths of time. The CRABPI message is expressed in AB1 stem cells, but is induced to much higher levels upon treatment with RA (Fig. 2). Induction of CRABPI gene expression is similar to the induction of “late” response genes since CRABPI message levels are still increasing 72 h after RA addition. Most intriguing, though, is that the CRABPI message is induced at low exogenous RA concentrations, but is almost extinguished by higher RA concentrations. CRABPI mRNA is induced to the greatest extent after AB1 cells have been exposed to 100 pM to 1 nM RA (Fig. 2). In contrast, CRABPII mRNA is expressed at increasingly higher levels as exogenous RA concentrations are raised, with maximal expression detected upon addition of 1 μM RA (Fig. 2).

We have also examined the expression of other RA-responsive genes such as Hox a1, Hox b1, collagen IV (α1), laminin B1, and tissue plasminogen activator. Thus far, all of the genes that have been examined appear to behave like the CRABPII gene; the genes are most highly induced at the highest RA concentrations (data not shown). These data suggest that the CRABPII gene is regulated by RA in a different manner from the CRABPI gene as well as from other RA-responsive genes.

Higher Levels of Functional CRABPI Protein Are Produced at Lower Exogenous RA Concentrations—We next wanted to determine if the increased level of CRABPI message expressed in cells treated with lower doses of RA resulted in increased production of functional CRABPI protein. AB1 cells were cultured in LIF and either 100 nM or 1 μM RA for 72 h. Cytoplasmic protein extracts were prepared from cells, incubated with [3H]RA, and resolved by nondenaturing polyacrylamide gel electrophoresis (PAGE) analysis (73, 74). The amount of functional CRABPI and CRABPII protein expressed in a particular cell line can be visualized by virtue of its ability to bind to [3H]RA. In addition, this procedure allows us to separate and directly compare the amount of CRABPI and CRABPII protein produced in a given cell line. The migration profiles demonstrate that significant amounts of functional CRABPI and CRABPII protein are produced in AB1 cells after treatment with 1 μM RA for 72 h (Fig. 3A). CRABPI protein levels are about 1.4-fold higher than CRABPII protein levels. After treatment with 100 nM RA (Fig. 3B), AB1 cells express an increased level of CRABPI protein and a decreased level of CRABPII protein when compared to the levels of CRABPI and CRABPII proteins expressed in cells treated with 1 μM RA (compare Fig. 3, A and B). This results in a 5.0-fold higher level of CRABPI than CRABPII at 100 nM RA. These data indicate that as the concentration of exogenously added RA is increased, CRABPII message and protein levels also increase while CRABPI message and protein levels decrease.

Increased Induction of CRABPI Expression at Low RA Concentrations Is Not a Result of Message Stabilization—To investigate whether the increased RA responsiveness of the CRABPII gene at low RA concentrations was a result of increased stability of CRABPI message, we determined the half-life of the CRABPI message in cells treated with high versus low exogenous RA.
The presence or absence of 1 nM RA concentrations. AB1 cells were cultured in LIF and in the presence of 1 μM or 100 nM RA for 72 h. 100 μg of cytoplasmic protein extract was incubated with 50 nM [3H]RA and samples were resolved on 12% nondenaturing polyacrylamide gels. Tritiated counts from 2-mm gel slices were quantitated by liquid scintillation counting. The results are expressed as the total number of 3H counts per min as a function of gel slice number, with gel slice 40 corresponding to the bottom of the gel. The two peaks corresponding to CRABPI and CRABPII protein are designated on the graph. These peaks represent proteins that bind specifically to RA since they can be eliminated when the protein extracts are incubated with an excess of unlabeled RA (data not shown). Gel slices 33–39 correspond to the peak of unbound [3H]RA, although the scales have been expanded so that the top of the peak of free [3H]RA is not shown. [3H]RA protein binding from AB1 cells grown in the presence of (A) 1 μM RA and (B) 100 nM RA are shown. This experiment was performed twice with similar results.

Retinoic Acid-induced Gene Expression in AB1 Cells

In the next series of experiments, we characterized AB1 cells for their ability to metabolize retinoic acid. Studies in our laboratory and by others have shown that many cell types, including other teratocarcinoma cells and embryonic stem cells, have the ability to metabolize RA to various polar derivatives (41, 83–85). In F9 teratocarcinoma cells, RA is metabolized relatively slowly in undifferentiated cells (85); however, the rate of RA metabolism is dramatically increased when F9 cells are cultured in the presence of 1 μM RA. Thus, RA can induce its own metabolism in F9 cells. Work in our laboratory has suggested that enzymes involved in the rapid metabolism of RA are induced by 8 h after RA treatment of F9 cells.

Most of the studies investigating RA metabolism in RA-differentiated cells have been performed after culturing cells in the presence of high exogenous RA concentrations (i.e. 1 μM RA) (85). Fewer studies have been done to analyze RA metabolism in cells cultured in the presence of varying concentrations of RA, and to identify possible qualitative differences in the types of polar RA metabolites generated.

AB1 cells were cultured in LIF in the absence or presence of 1 μM or 1 nM RA for 72 h, followed by exposure to 5 or 50 nM [3H]RA for 1 or 2 h. After labeling, cells and media were harvested, retinoids were extracted, and [3H]RA metabolites were resolved by HPLC. Non-radiolabeled retinoid standards were added to each sample to permit the identification of some of the radiolabeled retinoids. The HPLC tracings of one such set of cell and media samples from a 2-h incubation with 50 nM [3H]RA are shown in Fig. 5A. After 2 h of incubation with [3H]RA, both undifferentiated and RA-differentiated AB1 cells take up and metabolize significant amounts of [3H]RA, as evidenced by the presence of a peak corresponding to RA and by the presence of a large number of [3H] polar RA derivatives inside the cell and in the culture medium (Fig. 5A). The identities of all of these polar [3H]RA metabolites are not known, although unlabeled standards for 4-oxo-RA and 4-hydroxy-RA co-elute with some of the peaks in the region of minute 10. A comparison of the types of [3H]RA polar metabolites produced in AB1 cells cultured in the presence of 1 μM or 1 nM RA does not reveal any qualitatively different metabolites between the two culture conditions. Rather, the differences among the three culture conditions are more quantitative in nature.

Analysis of multiple samples of both [3H]retinoids extracted from cells and from the media at different times after the addition of [3H]RA allowed the kinetics of [3H]RA metabolism to be determined. The total RA concentration for each time point was calculated by using the following formula:

\[
\text{Total [RA]} = \frac{\text{cpm}_{\text{cell}} + 4\text{cpm}_{\text{med}}}{\text{cpm}_{\text{H]}[\text{RA}\rangle}} \times 5 \text{ nM (or 50 nM)} \quad \text{(Eq. 1)}
\]

where \(\text{cpm}_{\text{cell}}\) = tritiated RA counts in the cell sample, \(\text{cpm}_{\text{med}}\) = tritiated RA counts in the media sample, and \([\text{H]}[\text{RA}\rangle\) = the number of tritiated cpm from [3H]RA in the medium at the start of the experiment prior to the addition of cells. Thus, the data in Fig. 5B are plotted as the total amount of [3H]RA versus time. The two panels depict the kinetics of [3H]RA metabolism in AB1 cells incubated in the presence of either 50 (left panel) or 5 nM [3H]RA (right panel). AB1 stem cells metabolize [3H]RA relatively quickly, such that [3H]RA has a \(t_{1/2}\) of about 2 h (Fig. 5B, open circles). After 72 h of culture in the presence of 1 μM RA, the rate of [3H]RA metabolism in AB1 cells is dramatically increased, such that [3H]RA has a \(t_{1/2}\) between 30 and 45 min (Fig. 5B, closed triangles). The rate of [3H]RA metabolism in AB1 cells cultured in the presence of 1 μM RA resembles that found in undifferentiated AB1 stem cells; the \(t_{1/2}\) of [3H]RA in both cases is approximately 2 h (Fig. 5B, closed squares). We also found that AB1 cells exhibited similar kinetics of [3H]RA

\(^3\) C. Achkar and L. J. Gudas, unpublished observations.
In summary, in AB1 cells grown in the absence of RA or in the presence of 1 nM RA for 72 h, [3H]RA had a t₁/₂ of 2–2.5 h. In AB1 cells grown in the presence of 1 μM RA for 72 h, [3H]RA exhibited a t₁/₂ of 40–45 min.

We next examined the kineticsof [3H]RA metabolism in AB1 cells cultured for 72 h in a greater range of exogenous RA concentrations (Fig. 5 C); this experiment is similar to that shown in Fig. 5 B, left panel, except that different cell samples were initially cultured in each of four different RA concentrations. In AB1 cells initially cultured in the absence of RA or in the presence of 1, 10, or 100 nM RA for 72 h, [3H]RA had a t₁/₂ of 2–2.5 h; in cells cultured for 72 h in the presence of 1 μM RA, [3H]RA exhibited a t₁/₂ of 40–45 min.

Thus, there is a striking increase in the rate of [3H]RA metabolism when AB1 cells are initially cultured in 1 μM RA as compared to lower concentrations of RA (Fig. 5 C).

In another series of experiments, a much higher concentration of [3H]RA (500 nM) was added to AB1 cells which had initially been cultured in the absence or presence of 1 μM RA for 48 h. Under these conditions the t₁/₂ of [3H]RA was greater both for cells grown in the absence or presence of 1 μM RA; the t₁/₂ values are >6 and 1.5 h, respectively (Table I). These results indicate that even when the enzyme(s) which metabolize the [3H]RA are greatly induced or activated by culturing cells in 1 μM RA, the enzymes can be saturated by high substrate concentrations; this results in a longer half-life for [3H]RA in the cells (Table I).

Intracellular RA Levels in AB1 Cells—The intracellular RA concentration is a function of the rate of [3H]RA uptake versus the rate of [3H]RA metabolism. Regardless of the culture conditions, AB1 cells take up large amounts of [3H]RA from the medium. When cells are labeled in medium containing 5 or 50 nM [3H]RA, the actual concentration of [3H]RA which is associated with the cells is actually in the micromolar range (from 0.5 to 4.0 μM) within 2 h after the addition of [3H]RA to the medium (Table I).

There are quantitative differences in the levels of [3H]RA present in cells cultured under various conditions (Fig. 5 A, Table I). AB1 cells cultured for 72 h in the absence of RA and then labeled with 5 nM [3H]RA have an intracellular [3H]RA concentration of about 1.6 μM (Table I, control), while cells initially cultured in the presence of 1 μM exogenous RA and then labeled have an intracellular [3H]RA concentration of about 500 nM (Table I, 1 μM RA). Cells grown in the presence of 1 nM RA for 72 h prior to labeling have an intracellular concentration of approximately 3.7 μM [3H]RA (Table I, 1 nM RA). These results indicate that undifferentiated AB1 stem cells and AB1 cells initially cultured in the presence of 1 nM RA contain significantly higher levels of intracellular [3H]RA 2 h after addition of the labeled RA than cells differentiated by initial culture for 72 h with 1 μM RA (Table I).

This data suggests that the uptake of [3H]RA by the cells occurs rapidly and that the metabolism of [3H]RA is rate-limiting in untreated AB1 stem cells and in cells initially cultured in the presence of 1 nM RA, presumably because the enzyme(s) which metabolize [3H]RA are not very active under these conditions. In contrast, AB1 cells initially cultured for 72 h in the presence of 1 μM exogenous RA exhibit a higher level...
Fig. 5. Kinetics of RA metabolism in AB1 cells. AB1 cells were cultured in LIF in the absence or presence of varying concentrations of RA for 72 h. Cells were then labeled with either 5 or 50 nM [3H]RA for differing lengths of time. Cells and one-quarter of the media were harvested, retinoids were extracted, and reverse-phase HPLC analysis was performed to separate the retinoids. Non-radiolabeled retinoids were included with each sample as standards to determine the elution times of various retinoids, including RA (data not shown). In Fig. 5A, the HPLC tracings of cell and media samples, prepared from AB1 cells cultured in the absence or presence of 1 μM or 1 nM RA for 72 h and then labeled for 2 h with 50 nM [3H]RA, are presented. The data for each sample is plotted as tritiated counts per min versus time. The peaks corresponding to [3H]all-trans-RA and [3H]-polar metabolites are indicated in the tracing. Please note that the scales of the y axes are different for the different samples. B, the kinetics of [3H]RA metabolism in AB1 cells cultured as described above, and then labeled with either 5 or 50 nM [3H]RA for various times. The data are plotted as total [3H]RA concentration versus time. To determine total RA concentration, we first calculated the sum of [3H]RA counts per min in the cells (C) and media (M) for each time point. Since only one-quarter of the media was assayed, the total [3H]RA counts were obtained by taking the sum of C × 4 M for each time point. Since the concentration of the starting [3H]RA was known, total [3H]RA counts can be converted to [3H]RA.
of enzymatic activity. Under these conditions, less [3H]RA accumulates in the cells since a greater proportion of the intracellular [3H]RA is metabolized (Table I). However, even when the enzyme(s) which metabolize [3H]RA are induced by 48 h of culture in 1 μM RA, a high concentration of [3H]RA, 500 nM, can saturate these metabolic enzymes and result in a high level of [3H]RA within the cells (Table II).

### Table I

| Initial culture condition prior to labeling with [3H]RA | Concentration of [3H]RA added to the medium of AB1 cells in monolayer culture |
|--------------------------------------------------------|-------------------------------------------------------------------------------|
| Control, no added RA                                   | 5 nM | 50 nM | 500 nM |
| 1 nM RA, 72 h                                          | 2.5 ± 0.5 | 2.0 ± 0.5 | >6.0 ± 0.5 |
| 10 nM RA, 72 h                                         | ND  | 2.0 ± 0.5 | ND  |
| 100 nM RA, 48 h                                        | ND  | 1.0 ± 0.5 | ND  |
| 100 nM RA, 72 h                                        | ND  | 1.8 ± 0.5 | ND  |
| 1 μM RA, 48 h                                          | ND  | 1.0 ± 0.5 | ND  |
| 1 μM RA, 72 h                                          | 40-45 min | 40-45 min | ND  |

*These t½ values for [3H]RA were obtained from multiple experiments; t½ values varied less than 20% in the different experiments and an average value is shown.

### Table II

| Culture condition prior to radiolabeling | Concentration of [3H]RA added to the medium of AB1 cells in monolayer culture |
|-----------------------------------------|-------------------------------------------------------------------------------|
| Control, no added RA                    | 5 nM | 50 nM | 500 nM |
| 1 μM RA, 72 h                           | 1.6 ± 0.5 | 0.5 ± 0.5 | >50 ± 0.5 |
| 1 nM RA, 72 h                           | 3.7 ± 0.5 | 2.3 ± 0.5 | ND  |
| 1 μM RA, 48 h                           | ND  | 0.4 ± 0.5 | 3.6 ± 0.5 |
| 1 μM RA, 72 h                           | 0.5 ± 0.5 | 0.2 ± 0.5 | ND  |

*ND, not determined.

These data are from three different experiments; an average value was calculated.

### Fig. 6: Formation of polar metabolites in AB1 cells

AB1 cells were cultured as described in Table I, then labeled for 1 or 2 h with 50 nM [3H]RA. Cells and media were harvested, retinoids were extracted, and reverse-phase HPLC analysis was performed. The data are plotted as tritiated counts per min of cell-associated polar metabolites or [3H]polar metabolites in the medium versus the culture condition over the prior 72 h. This experiment was performed three times with similar results; one representative experiment is shown. The data presented in this figure were obtained from the same experiment as the data presented in Fig. 5B, left panel.
medium (Fig. 6, bottom panel, open bars, 10^{-6} M RA) than AB1 stem cells (Fig. 6, top and bottom panels, open bars, stem). Since the kinetics of [3H]RA metabolism by AB1 stem cells versus AB1 cells cultured 72 h in 1 nM RA are similar (Fig. 5B and C), we conclude that the levels of [3H]-polar metabolites are higher within AB1 cells initially cultured in 1 nM RA because a greater proportion of the polar metabolites remains in these cells than in AB1 stem cells. Under these same culture conditions (72 h of culture in the presence of 1 nM RA), the CRABPI mRNA level is very high (Fig. 2). Therefore, one potential explanation for the high intracellular levels of [3H]-polar metabolites under these conditions is that the polar metabolites are bound by CRABPI protein in the cells. This explanation is consistent with data from Fiorella and Napoli (88). There researchers previously demonstrated that 4-hydroxy-RA and 4-oxo-RA, when bound to CRABPI, were metabolized very slowly in testis microsomal extracts; in contrast, unbound 4-hydroxy-RA and 4-oxo-RA had elimination t\textsubscript{1/2} values of 40 and 9 min, respectively.

**DISCUSSION**

RA-responsive Gene Expression—AB1 murine embryonic stem cells differentiate into extraembryonic, parietal endoderm-like cells in response to RA treatment (Fig. 1). As such, these cells behave similarly to RA-responsive teratocarcinoma and other embryonic stem cell lines characterized previously. All of the differentiation-specific genes examined in this paper have been characterized previously in other RA-responsive cell culture models as being RA-responsive genes (2, 6, 65, 67–69, 78, 80). These include both early response genes, such as homeobox genes (Hox a1 and Hox b1) and zinc-finger AB1 gene in AB1 cells cultured in the presence of low concentrations of RA. After culture in the presence of high exogenous RA concentrations, AB1 cells exhibited a dramatically increased rate of [3H]RA metabolism to [3H]-polar derivatives (Fig. 5; Table I). In contrast, AB1 cells cultured in 1 nM RA did not show an increase in the rate of [3H]RA metabolism; they metabolized [3H]RA at about the same rate as stem cells (Fig. 5; Table I). From the experiments reported here it is clear that in AB1 cells, RA induces its own metabolism to polar metabolites via a high capacity, low affinity enzyme system. These polar metabolites are then further metabolized via an inducible enzyme system. The uptake of RA appears to occur by a rapid, high capacity mechanism such that under certain conditions, e.g., in AB1 stem cells, the enzyme(s) which metabolize RA are rate-limiting and large quantities of RA accumulate in the cells. Some information is available concerning the enzymes which metabolize RA, and it is known that a number of cytochrome P450 enzymes can metabolize RA (89–96). Reynolds et al. (97) have shown that metabolism of RA occurs in human keratinocytes, but the enzyme(s) involved have not been thoroughly studied. Various purified isozymes of microsomal cytochrome P450 have been shown to be capable of metabolizing RA and other retinoids (98). The identities of the enzyme(s) involved in RA metabolism in these embryonic cells are unknown.

One of the most striking results of this study is that the actual intracellular [3H]RA concentrations are extremely high when AB1 cells are cultured in the presence of 1 nM to 1 \mu M exogenous RA in the medium (Table II). How much of this cell-associated RA is associated with intracellular membranes versus bound to proteins such as CRABPI, CRABPII, and the RARs is not known. The K\textsubscript{d} of CRABPI for RA has previously been determined to be about 7 nM (38, 39, 99). Thus, the intracellular RA concentration would be far above the K\textsubscript{d} of CRABPI even when these AB1 cells are cultured in the presence of nanomolar concentrations of exogenous RA (Table II). It would be expected that virtually all of the CRABPI protein in the cell would be bound to RA, and that most of the RA in the cell would be present as free RA or RA bound to membranes and/or other proteins.

We have made similar observations concerning the high intracellular RA levels in other cell types cultured in the presence
of exogenous RA,4 so this result is not unique to AB1 cells. These results have important implications for studies in which cells or tissues are cultured in the presence of high exogenous concentrations of RA. The local concentrations of RA required to elicit some biological responses may be higher than previously thought, based on the exogenous concentrations of RA used to elicit biological effects. Furthermore, such high intracellular concentrations of RA may lead to the activation of extraneous receptors not normally activated by endogenous retinoids. The fact that RA is so highly teratogenic may also be explained in part by this ability of cells to accumulate RA from the surrounding medium to extremely high intracellular levels.

Acknowledgments—We thank Dr. John Wagner for critically reading this manuscript, Dr. Jochen Buck for advice about the HPLC system, and Taryn Resnick for editorial assistance.

REFERENCES

1. Sporn, M. B., and Roberts, A. B. (1983) Cancer Res. 43, 3034–3040
2. Gudas, L. J., Sporn, M. B., and Roberts, A. B. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 443–520, Raven Press Ltd., New York
3. Eichele, G. (1989) Trends Genet. 5, 246–251
4. Hohenester, E., and Eichele, G. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 387–442, Raven Press Ltd., New York
5. Fuchs, E., and Green, H. (1981) Cell 25, 617–625
6. Gudas, L. J. (1991) J. Biol. Chem. 266, 171–179
7. Jetten, A. M., Jetten, M. E., Shapiro, S. S., and Poon, J. P. (1979) Cell 19, 287–299
8. Jetten, A. M., Kim, J. S., Sacks, P. G., Rearick, J. I., Lotan, D., Hong, W. K., and Lotan, R. (1990) Int. J. Cancer 45, 195–202
9. Lotan, R., and Lotan, D. (1981) J. Cell. Physiol. 106, 179–189
10. Pieck, G. L., and DiGiovanna, J. J. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 631–658, Raven Press Ltd., New York
11. Hill, D. L., and Grubbs, C. J. (1992) Annu. Rev. Nutr. 12, 161–181
12. Hong, W. K., and Jetten, L. M. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 659–685, Raven Press Ltd., New York
13. Castaigne, S., Chomienne, C., Daniel, M. T., Ballerini, P., Berger, R., Fenaux, S., and Degos, L. (1990) Blood 76, 1704–1709
14. Chomienne, C., Ballerini, P., Baltrán, N., Daniel, M. T., Fenaux, P., Castaigne, S., and Degos, L. (1990) Blood 76, 1710–1717
15. Hong, W. K., Lippman, S. M., Itri, L. M., Karp, D. D., Lee, J. S., Byers, R. M., Schantz, S. P., Kramer, A. M., Lotan, R., Peters, L. J., Dimery, I. W., Brown, B. W., and Goepfert, H. (1990) N. Engl. J. Med. 323, 795–801
16. Lampero, E. K., Hoar, R. P. M., Agostini, J., Braun, J. T., Curry, C. J., Ferroh, P. M., Grix, A. W., Lott, T. T., Richard, J. M., and Sun, S. C. (1985) N. Engl. J. Med. 313, 837–841
17. Sulik, K. K., Cook, C. S., and Webster, W. S. (1988) Development 103, suppl. 213–232
18. Armstrong, R. B., Ashenfelter, K. O., Echhoff, C., Levin, A. A., and Shapiro, S. S. (1981) Nature 294, 383–389
19. Levin, A. A., Sporn, M. B., and Roberts, A. B. (1983) in Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 545–552, Raven Press Ltd., New York
20. Warrell, R. P., Jr., de Thé, H., Wang, Z.-Y., and Degos, L. (1993) Nature 368, 177–180
21. Mageolsdorff, D. J., Thummler, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
22. Mageolsdorff, D. J., Umesono, K., and Evans, R. M. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 319–350, Raven Press Ltd., New York
23. Bugge, T. H., Pohl, J., Loonoy, O., and Stunnenberg, H. G. (1992) EMBO J. 11, 1409–1418
24. Hecht, A., and Mageolsdorff, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992) Cell 68, 397–406
25. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S., and Chambon, P. (1992) Cell 68, 377–395
26. Levin, A. A., Sturzenback, L., Kazmier, S., Bosakowski, T., Huselson, C., Allenby, G., Speck, J., Kratzesc, C. L., Rosengren, M., Lovey, A., and Grillo, J. F. (1994) Nature 371, 391–396
27. Yu, R. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Năã, A. M., Kim, S. Y., Boutin, J. M., Glass, C., and Rosenfeld, M. G. (1991) Cell 68, 323–336
28. Zhang, J.-K., Hoffmann, B., Tran, P.-V., Graupner, G., and Pfahl, M. (1992) Nature 355, 441–444
29. de Thé, H., van Vrijenhuizen, T. M. D., Vlidi, P., Stunnenberg, H., and Dejean, A. (1986) Nature 324, 173–175
30. Langston, A. W., and Gudas, L. J. (1992) Mech. Dev. 38, 217–228
31. Smith, W. C., Nakshatri, H., Leroy, P., Rees, J., and Chambon, P. (1991) EMBO J. 10, 2223–2230

4 A. C. Chen and L. J. Gudas, unpublished data.
Retinoic Acid-induced Gene Expression in AB1 Cells

84. Gubler, M. L., and Sherman, M. I. (1985) J. Biol. Chem. 260, 9552–9558
85. Williams, J. B., and Napoli, J. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4658–4662
86. Bloom, W., and Fawcett, D. W. (eds) (1975) A Textbook of Histology, 10th Ed., W. B. Saunders Co., Philadelphia, PA
87. Keith, C., Di Paola, M., Maxfield, F. R., and Shelanski, M. L. (1983) J. Cell Biol. 97, 1918–1924
88. Fiorella, P. D., and Napoli, J. L. (1994) J. Biol. Chem. 269, 10538–10544
89. Van Wauwe, J. P., Coene, M. C., Goossens, J., Van Nijen, G., Cools, W., and Lauwers, W. (1988) J. Pharmacol. Exp. Ther. 245, 718–722
90. Martini, R., and Murray, M. (1993) Arch. Biochem. Biophys. 303, 57–66
91. Vanden Bossche, H., Willemsens, G., and Janssen, P. A. J. (1988) Skin Pharmacol. 1, 176–185
92. Van Wauwe, J. P., Coene, M. C., Goossens, J., Van Nijen, G., Cools, W., and Lauwers, W. (1988) J. Pharmacol. Exp. Ther. 245, 718–722
93. Barua, A. B., Gunning, D. B., and Olson, J. A. (1991) Biochem. J. 277, 527–531
94. Van Wauwe, J. P., Coene, M. C., Goossens, J., Cools, W., and Montaliu, J. (1990) J. Pharmacol. Exp. Ther. 252, 965–969
95. Frolik, C. A., Roller, P. P., Roberts, A. B., and Sporn, M. B. (1980) J. Biol. Chem. 255, 8057–8062
96. Bhat, P. V., and Jetten, A. M. (1987) Biochim. Biophys. Acta 922, 18–27
97. Reynolds, N. J., Fisher, G. J., Griffiths, C. E., Tavakkol, A., Talwar, H. S., Rowse, P. E., Hamilton, T. A., and Voorhees, J. J. (1993) J. Pharmacol. Exp. Ther. 266, 1636–1642
98. Roberts, E. S., Vaz, A. D. N., and Coon, M. J. (1992) Mol. Pharmacol. 41, 427–433
99. Grippo, J. F., and Gudas, L. J. (1987) J. Biol. Chem. 262, 4492–4500
An Analysis of Retinoic Acid-induced Gene Expression and Metabolism in AB1 Embryonic Stem Cells
Anne C. Chen and Lorraine J. Gudas

J. Biol. Chem. 1996, 271:14971-14980.
doi: 10.1074/jbc.271.25.14971

Access the most updated version of this article at http://www.jbc.org/content/271/25/14971

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 90 references, 45 of which can be accessed free at
http://www.jbc.org/content/271/25/14971.full.html#ref-list-1