5-Azacytidine and sodium butyrate induce expression of aromatase in fibroblasts from chickens carrying the henny feathering trait but not from wild-type chickens

(Sebright bantam/enzyme induction/estrogen synthesis/cytochrome P-450)

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ABSTRACT Male chickens with the henny feathering trait have a female feathering pattern. In two henny-feathered breeds, the Sebright bantam and the Golden Campine, the synthesis of estrogen is increased as a consequence of increased activity of aromatase, a cytochrome P-450 enzyme that converts androgen to estrogen. The activity of the enzyme is elevated in tissue slices and in cultured fibroblasts from heterozygous and homozygous birds of both breeds. In contrast, aromatase activity is very low in extraglandular tissues from control chickens and is undetectable in fibroblasts cultured from these tissues. The current studies show that two agents known to alter gene expression—5-azacytidine and sodium butyrate—markedly induce expression of aromatase activity in Sebright and Campine fibroblasts but have no effect on aromatase activity in fibroblasts from wild-type chickens. Induction of aromatase is specific since two other microsomal enzymes in chicken fibroblasts—one, a component of the aromatase enzyme complex and the other a cytochrome P-450 oxidase distinct from the aromatase—are not significantly affected by these agents. Further study of this unique mutation should provide insight into the mechanisms by which genes are switched to an inducible state during differentiation.

Aromatase is a cytochrome P-450 oxidase that converts androgens, such as testosterone and androstenedione, to estrogen (1). The expression of the enzyme is tissue specific to some degree in all species, and this tissue specificity is particularly striking in the chicken (2). In most breeds of chickens aromatase is expressed only in the ovaries and cannot be detected in any other tissue of the female or in any tissue of the male either during embryogenesis or later in development (2,3).

In two breeds of chicken, the Sebright bantam and the Golden Campine, the tissue-specific restriction of aromatase is disrupted (2,4). In these birds aromatase is expressed in many tissues of both males and females beginning at the fourth day of embryonic development (3). High enzyme levels persist in several tissues during adulthood, primarily in the skin. This increase in aromatase activity is due to an increase in the activity of the terminal cytochrome P-450 oxidase component of the aromatase enzyme complex (5). Fibroblasts cultured from a number of tissues of Sebright chickens express aromatase, whereas fibroblasts from wild-type chickens do not (4). The enzyme that is expressed in peripheral tissues and fibroblasts of Sebright and Campine chickens is indistinguishable by kinetic analyses from the aromatase of normal ovary (6).

As a result of aromatase expression in skin, male Sebright and Campine birds develop a female feathering pattern (7) known as the henny feathering trait. This trait was studied by Morgan, who concluded that it was the result of an autosomal mutation (8). More recent studies have confirmed that this trait is due to a single autosomal gene mutation; homozygous carriers of the gene express full activity of aromatase in skin, and heterozygous carriers have intermediate levels of activity (9). These observations suggest that the mutant gene prevents normal suppression of aromatase in nonovarian tissues. Understanding the nature of this mutation might provide insight into the normal process by which tissue-specific control of enzyme activity takes place.

A variety of techniques have been utilized to study the mechanism of tissue-specific gene expression both in vitro systems and in intact animals. For example, genes that are not expressed in specific tissues are usually more extensively methylated than they are in tissues in which the gene is expressed, and prevention of methylation in growing cells can result in expression of previously dormant genes (10). Addition of 5-azacytidine, an inhibitor of DNA methylation, to embryonic mouse fibroblasts in culture elicits a program of differentiation into myocytes that requires the expression of many genes (11). Since many genes are not induced by 5-azacytidine the concept has arisen that expression of genes may involve demethylation as one part of a multistep mechanism. Hypomethylation of DNA may cause gene expression only when other unknown factors are present. Another pharmacological agent that alters gene expression in vitro is butyric acid. This compound has diverse actions, a major part of which may be the result of hyperacylation of chromosomal histones (12,13). In some systems 5-azacytidine and butyric acid act synergistically to induce gene expression (14, 15).

In the current experiments 5-azacytidine and sodium butyrate have been utilized in cultured fibroblasts from Sebright and Campine chickens to investigate the abnormal differentiation pattern of aromatase. 5-Azacytidine and sodium butyrate, alone and in combination, cause a profound increase in aromatase activity in fibroblasts cultured from chickens carrying the henny feathering trait but have no effect on expression of the enzyme in cells from normal birds. These findings suggest that the underlying mutation responsible for the henny feathering trait does not involve DNA methylation or histone acetylation but does permit increased expression of the aromatase gene when DNA methylation and histone acetylation are altered.

MATERIALS AND METHODS

Materials. Adult Silver Sebright bantam, White Leghorn bantam, and Golden Campine chickens were obtained from the Halbach Poultry Farm, Waterford, WI, and fertile eggs from Silver Sebright and game bantam chickens were obtained from David Sherrill, Arlington, TX. [1β-3H]Testosterone (18.5 Ci/mmol; 1 Ci = 37 GBq) was prepared from [1β,2β-3H]testosterone (46 Ci/mmol) (New England Nu-
clear) as described (4). Celite analytical filter was from Fisher, and charcoal (Norit) was from Mallinckrodt. Dulbecco’s modified Eagle’s medium, medium 199, chicken serum, and fetal calf serum were from GIBCO. 5-Azacytidine and sodium butyrate were from Sigma.

Culture of Fibroblasts and Preparation of Fibroblast Membranes. Fibroblasts were propagated as described (4) from biopsies of skin and breast muscle taken from adult chickens and of skin, heart, skeletal muscle, and lung removed from day 18 chicken embryos.

Culture dishes with cells grown for enzyme assay contained 10 ml of DMEM-199 with 5% chicken serum and 5% fetal calf serum. Additions of 5-azacytidine were made to subconfluent monolayer cultures on day 1, and on day 2 the medium was removed and fresh medium containing sodium butyrate was added. Cells were in logarithmic growth phase during the treatment period. On day 3 the fibroblast monolayers were rinsed twice with 3 ml of 50 mM Tris-HCl/50 mM NaCl, pH 7.4 (Tris/saline). Cells were scrapped into 2 ml of Tris/saline and a membrane fraction was prepared as described (4).

Assays. The standard assay of aromatase activity in fibroblast and ovarian membranes measured the release of $^3$H$_2$O from [1$^\beta$-$^3$H]testosterone during the conversion of androgen to estrogen by a previously described modification (4) of the procedure of Thompson and Siiteri (1).

NADPH-cytochrome c reductase was measured by a modification of the method of Masters et al. (16) as described (4). Membranes were resuspended in 10 mM Tris-HCl (pH 7.4) with a Dounce homogenizer. NADPH-cytochrome c reductase was calculated as a function of the change in absorbance at 550 nm at room temperature.

Steroid 21-hydroxylase was assayed by utilizing membranes prepared as described above and subsequently washed and resuspended in 10 mM Tris-HCl (pH 7.4). Enzyme activity was assayed by measuring the formation of 11-deoxycorticosterone from progesterone. Assay mixtures contained 1 $\mu$M [1,2,6,7-$^3$H]progesterone, 0.1 M Tris/citrate buffer (pH 7), 1 mM NADPH, 0.25 $\mu$M 11-deoxycorticosterone, and washed membranes (0.04–0.3 mg of protein) in a total volume of 0.2 ml. Incubations were at 25°C for 1 hr, and steroids were extracted with 1 ml of ethyl acetate. Nonradioactive 11-deoxycorticosterone (50 $\mu$g) was added, and radioactive 11-deoxycorticosterone was purified as the free steroid by three sequential thin-layer chromatography procedures utilizing the systems ethyl acetate/isooctane (70:30), methylene chloride/ethyl ether (80:20), and ethyl acetate/isooctane/acetic acid (45:45:10). In some experiments the validity of this procedure was confirmed by acetylation of the final product, mixing with deoxycorticosterone acetate, and demonstration that the ratio of $^3$H to $^{14}$C did not change following recrystallization.

Proteins were assayed by the method of Lowry et al. (17) with bovine serum albumin as the standard.

RESULTS

Addition of 5-azacytidine to actively dividing monolayer cultures of Sebright and Campine skin fibroblasts resulted in a 6- and 17-fold stimulation of aromatase activity, respectively (Fig. 1A and B). Similar results were observed with fibroblasts obtained from both male and female Sebright chickens (results not shown). No stimulation of aromatase was observed in control skin fibroblasts that were treated in the same way. Comparable degrees of stimulation were observed in whole homogenate, whole cell, and membrane assays (results not shown). A variable toxic effect of the 5-azacytidine on cell growth was concentration-dependent (Fig. 1C), but this inhibition was negligible with amounts that produced maximal stimulation of aromatase. In all embryonic Sebright fibroblasts examined in skin fibroblasts from adult Sebrights maximal stimulation of aromatase activity was observed by utilizing 0.25 or 0.5 $\mu$M 5-azacytidine, whereas maximal stimulation occurred at 1 and 2.5 $\mu$M 5-azacytidine in the Campine skin fibroblasts and adult Sebright muscle fibroblasts, respectively. An effect on aromatase activity was observed within 6 hr of exposure to 5-azacytidine but maximal stimulation required exposure for 24 hr. Following removal of 5-azacytidine from the culture medium, stimulation of enzyme activity persisted for as long as 6 days (results not shown).

The addition of sodium butyrate to cultures of Sebright and Campine skin fibroblasts resulted in a 2- and 2.5-fold stimulation of aromatase activity, respectively, but was without effect on aromatase in control fibroblasts (Fig. 2A and B). In contrast to 5-azacytidine, little or no toxic effect on cell growth or protein was observed with the concentrations of butyrate examined (Fig. 2C). Stimulation of aromatase in-

![Fig. 1](image-url)

**Fig. 1.** Effect of 5-azacytidine on aromatase activity. 5-Azacytidine was added to cultures of adult skin fibroblasts 1 day after plating; on day 2 the medium was replaced with fresh medium containing 5-azacytidine, and on day 3 membranes were prepared and assayed for aromatase activity and protein. (A) Aromatase activity, pmol/hr/dish. (B) Aromatase activity, pmol/hr/dish/(mg of membrane protein)$^{-1}$. (C) Membrane protein, $\mu$g per dish. ●, Sebright fibroblasts; ○, Campine fibroblasts; □, control fibroblasts.
creased with increasing concentrations of butyrate between 2.5 and 10 mM.

The magnitude of stimulation of aromatase in Sebright fibroblasts sequentially exposed to 5-azacytidine and butyrate was greater than with either agent alone. In the experiment shown in Fig. 3 exposure of adult Sebright skin fibroblasts to 0.25 μM 5-azacytidine resulted in an increase in aromatase specific activity from 4.1 to 21.8 pmol/hr⁻¹(mg of protein)⁻¹, and exposure to 10 mM butyrate stimulated aromatase activity to 17.3 pmol/hr⁻¹(mg of protein)⁻¹. The sequential treatment with 5-azacytidine followed by sodium butyrate resulted in an aromatase activity of 70.2 pmol/hr⁻¹(mg of protein)⁻¹. Simultaneous exposure of cells to both 5-azacytidine and butyrate during the final day of cell growth resulted in a markedly decreased enhancement of aromatase activity (results not shown). Inclusion of either 5-azacytidine (0.5 and 1 μM) or sodium butyrate (5 and 10 mM) in the aromatase assay itself had no effect (results not shown). In the Campine fibroblasts, addition of butyrate to 5-azacytidine-exposed cells did not enhance aromatase activity above the level seen with 5-azacytidine alone (results not shown).

A comparison of the effect of these agents on aromatase activity in fibroblasts derived from skeletal muscle of Sebright chickens of different ages is shown in Table 1. Basal enzyme activity is lower in adult than in embryonic fibroblasts, but following treatment with 5-azacytidine and butyrate aromatase activity increases to a similar level in both strains. Sequential addition of 5-azacytidine and butyrate consistently stimulated aromatase activity in fibroblasts from several embryonic Sebright tissues—skin, skeletal muscle, heart, and lung (Table 2). Because of higher basal activity in these fibroblast strains, the degree of stimulation (3- to 4-fold) was lower than with the adult and embryonic skeletal muscle strains described in Table 1. Again, no stimulation of aromatase by these agents was observed in fibroblasts derived from embryonic control skin or muscle.

The specificity of the stimulatory effect of 5-azacytidine and butyrate on the androgen-binding cytochrome P-450 oxidase component of the aromatase enzyme complex was
The regulation of aromatase activity in tissues of the chicken constitutes a striking example of the genetic control of enzyme differentiation. In the normal male chicken the enzyme is not measurable in any tissue, beginning as early as the fourth day of embryogenesis and continuing through the adult life of the bird. In the normal female, during the same lifespan, the enzyme is expressed only in the ovary (2, 3).

Sebright bantam and Campine chickens of both sexes have an autosomal mutation termed the henny feathering trait (9) that causes expression of the aromatase enzyme in many tissues from at least the fourth day of embryogenesis (3) and that causes affected males to develop a female feathering pattern (7). The mutation does not completely prevent suppression of aromatase activity since with advancing development enzyme activity in these birds does decline to low levels in tissues other than ovary, skin, and skin appendages (2, 3). Elucidation of the nature of the abnormal control of enzyme expression in the henny-feathered chicken may provide insight into the process by which selective restriction of enzyme activity normally prevents expression in tissues other than ovary.

To explore the nature of this mutation, the effects on aromatase activity of two agents known to alter gene expression were examined in fibroblasts cultured from normal chickens and from chickens carrying the henny feathering trait. The protocol utilized was originally used to demonstrate induction of β-adrenergic receptors in HeLa cells and involves the sequential addition of 5-azacytidine followed by sodium butyrate (14). The primary effect of 5-azacytidine is inhibition of DNA methylation (10), whereas, among other effects, sodium butyrate has the ability to inhibit deacetylation of histones (13). However, both compounds have multiple actions and alter the expression of many but not all genes both in vivo and in vitro (10, 13).

In these studies it has been demonstrated that (i) aromatase activity can be enhanced only in fibroblast strains from animals carrying the henny trait, (ii) the maximal level of induced activity is similar in fibroblasts derived from diverse tissues and is independent of the basal level, (iii) the degree of induction (over baseline) but not the absolute level of activity is greater in fibroblasts derived from tissues of mature chickens than in fibroblasts derived from embryonic tissues, and (iv) the induction is relatively specific in that the other cytochrome P-450 enzyme tested was not influenced by these agents. Similar results were obtained in two breeds of chicken carrying the henny trait (Sebright bantam and Golden Campine).

The observation that 5-azacytidine and butyrate are ineffective in inducing aromatase activity in the control fibroblasts indicates that the normal aromatase gene in extravascular cells is in a state that renders it impervious to the effects of these agents. The mutation that gives rise to the henny feathering trait has two consequences. First, it impairs the mechanism that normally restricts expression of the gene in extravascular tissues. Second, the mutation renders the gene susceptible to regulation by agents that inhibit DNA methylation and histone deacetylation, processes that do not influence gene expression in the normal chicken. It seems reasonable to speculate that the mutation impairs some

**Table 1. Effect of 5-azacytidine and butyrate on aromatase activity in fibroblasts grown from adult and embryonic Sebright skeletal muscle**

<table>
<thead>
<tr>
<th>Source of fibroblast membranes</th>
<th>Aromatase, pmol·hr⁻¹·(mg of protein)⁻¹</th>
<th>5-Azacytidine and butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Sebright skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult Campine skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult control skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control embryos</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5-Azacytidine (2.5 μM) and butyrate (10 mM) were added according to the standard protocol. On day 3 fibroblasts were prepared and assayed for aromatase.

**Table 2. Effect of 5-azacytidine and butyrate on aromatase activity in fibroblasts from day 18 embryonic chicken tissues**

<table>
<thead>
<tr>
<th>Source of fibroblast membranes</th>
<th>Aromatase, pmol·hr⁻¹·(mg of protein)⁻¹</th>
<th>5-Azacytidine and butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sebright embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>74</td>
<td>210</td>
</tr>
<tr>
<td>Muscle</td>
<td>58</td>
<td>168</td>
</tr>
<tr>
<td>Heart</td>
<td>20</td>
<td>82</td>
</tr>
<tr>
<td>Lung</td>
<td>84</td>
<td>229</td>
</tr>
<tr>
<td>Control embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Muscle</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

Fibroblasts were propagated and cultured from day 10 Sebright and control chicken embryos. 5-Azacytidine (0.25 μM) was added 1 day after plating, and butyrate (10 mM) was added 2 days after plating by the standard protocol. On day 3 membranes were prepared and aromatase activity was assayed.

**Table 3. Effect of 5-azacytidine and butyrate on aromatase, steroid 21-hydroxylase, and NADPH-cytochrome P-450 reductase activities in fibroblast membranes**

<table>
<thead>
<tr>
<th>Source of fibroblast membranes</th>
<th>Aromatase, pmol·hr⁻¹·(mg of protein)⁻¹</th>
<th>Steroid 21-hydroxylase, pmol·hr⁻¹·(mg of protein)⁻¹</th>
<th>NADPH-cytochrome P-450 reductase, nmol·min⁻¹·(mg of protein)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Sebright skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 18 embryonic Sebright heart</td>
<td>21</td>
<td>127</td>
<td>2.0</td>
</tr>
<tr>
<td>Adult Campine skin</td>
<td>20</td>
<td>82</td>
<td>4.2</td>
</tr>
<tr>
<td>Adult control skin</td>
<td>0.2</td>
<td>3.4</td>
<td>4.6</td>
</tr>
<tr>
<td>Control skin</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
<td>1.8</td>
</tr>
</tbody>
</table>

5-Azacytidine (0.25 μM for Sebright and control fibroblasts) and butyrate (10 mM) were added according to the standard protocol. On day 3 fibroblast membranes were prepared and assayed for aromatase, steroid 21-hydroxylase, and NADPH-cytochrome P-450 reductase activities. –, No addition; +, 5-azacytidine and butyrate added.
primitive control mechanism that is fundamental to tissue-specific enzyme differentiation. The precise nature of the mutation is unclear. Possibilities include a gene duplication, a chromosomal rearrangement, or an unrecognized mutation of the structural gene for the enzyme that interferes with the normal suppression mechanism.

The finding that 5-azacytidine and butyrate cause a similar maximal degree of enhancement of aromatase activity in Sebright fibroblasts derived from multiple tissues and from birds of different ages may explain two previously puzzling aspects of estrogen physiology. The first concerns the observation that enzyme activity declines with age in all extraovarian tissues of the Sebright chicken so that in the adult animal aromatase activity is high only in skin and skin appendages (3). Since the enzyme is stimulated by 5-azacytidine to a similar degree in fibroblasts from different tissues of the Sebright, it is possible that DNA methylation plays an important role in this secondary suppression mechanism. The second issue to which these findings may pertain involves the regulation of extraovarian estrogen production in the mammal. Tissue-specific restriction of aromatase activity to the mammalian ovary and placenta is never as absolute as ovarian restriction in the chicken. Thus, in mammals of both sexes (including the human), aromatase is detectable in small amounts in many extraovarian tissues, including adipose tissue, hair follicle, and skin (and in fibroblasts derived from normal skin) (18, 19). Furthermore, in the rabbit aromatase is transiently expressed in brain during embryogenesis; this activity may play a critical role in brain development (20). Such temporary expression of gene activity in the embryonic rabbit brain as well as the persistent expression in adipose tissue, skin, and skin appendages of all mammals may also involve processes susceptible to pharmacological manipulation by 5-azacytidine or butyrate (or both). A further understanding of the mechanism underlying the sensitivity of aromatase to 5-azacytidine and butyrate in Sebright and Campine chickens may help elucidate the mechanisms for tissue-specific restriction of gene expression. Knowledge of the structure of the aromatase gene and its flanking regions in Sebright and Campine chickens as compared with wild-type chickens should help reveal these mechanisms.

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