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# CHANGES IN GENE EXPRESSION DURING SENESCENCE OF ADRENOCORTICAL CELLS IN CULTURE

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**Summary**—Bovine adrenocortical cells undergo a process in which expression of steroid hydroxylases is lost progressively as a function of population doubling level (PDL) in culture. Each cytochrome *P*450 shows a characteristic rate of loss of expression as a function of PDL (in order of rates of loss: *CYP11B* > *CYP21* > *CYP17* > *CYP11A*). *CYP11B* and *CYP21* require insulin-like growth factor I as well as cyclic AMP; these are the only factors required for induction in the primary culture. Middle- and later passage cells do not express *CYP11B* and *CYP21* under the same conditions, but will do so when cells are grown in extracellular matrix Matrigel. In late-passage cells neither *CYP17*, *CYP21*, nor *CYP11B* are expressed, even in the presence of Matrigel; only *CYP11A* is expressed in late-passage cultures. When the different environmental factors required for induction of *CYP11B* and *CYP21* are taken into account, induction of these genes disappears with the same kinetics as previously shown for *CYP17* as a function of PDL. The primary cause of the loss of expression of these genes is likely to be a phenotypic switching event similar to that previously demonstrated for *CYP17* by *in situ* hybridization. The mechanism of phenotypic switching is unknown. However, one *Hpa*II site at −2.3 kb of *CYP17* was methylated in the bovine adrenal cortex *in vivo* but showed rapid and complete demethylation when adrenocortical cells were placed in culture. This indicates a unique, reproducible, environmentally determined change in methylation, with as yet undetermined consequences. However, data from reporter constructs suggest that phenotypic switching does not result from a simple loss of regulatory factors that act within 2.5 kb of the promoter. Previous data suggested that SV40 T antigen may affect phenotypic switching, and thus that SV40 may be useful for the derivation of functional adrenocortical cell lines. Adaptation of methods previously used for bovine cells to human adrenocortical cells to produce SV40 T antigen-transfected clones yielded data indicating preservation of essential aspects of the human adrenocortical cell differentiated phenotype.

OUTLINE



## 1. LONG-TERM GROWTH AND SENESCENCE OF BOVINE ADRENOCORTICAL CELLS

We have used a differentiated endocrine cell type to investigate mechanisms of cellular sensecence. The well-characterized inducible differentiated function genes of the adrenal cortex can be used to study the relationship between cellular senescence and the expression of differentiated properties [reviewed in 1, 2]. Most of our work has been performed on bovine adrenocortical cells, which offer several practical advantages over human adrenocortical cells for these studies, one being a very long *in vitro* "life span" (Table 1). An overall scheme for the use of adrenocortical cells in such experiments is shown in Fig. 1.

Cells isolated from the adrenal cortex and placed in primary culture are fully differentiated, as shown by the uniform expression of differentiated function genes [3], yet are capable of proliferation, as shown by the uniform incorporation of [$^3$H]thymidine into nuclei (Fig. 2). Fibroblast growth factor (FGF) is required for long-term cell proliferation or for clonal growth

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Table 1. Adrenocortical cell replicative potential in culture

| Species |
|---|
| Human |
| 30–40 PDL (fetal): 0–30 PDL (adult) |
| no spontaneous transformation |
| SV40 T antigen extends life span |
| Bovine |
| 100–120 PDL |
| no spontaneous transformation |
| SV40 T antigen extends life span |
| Rat |
| 20–30 PDL |
| ? spontaneous transformation |
| SV40 T antigen immortalizes |

The numbers under Species indicate the population doubling level (PDL) at which the replicative capacity of the cells is completely lost.

(Fig. 2). In the presence of FGF cell proliferation continues for 100 to 120 population doublings. During this long period of division two kinds of changes in differentiated function occur. The first results from the development of deficiencies of substances that were presumably present in the *in vivo* environment. The second results from longer term changes which may be related to cellular aging. In this later phase changes in the expression of differentiated function genes are observed as phenotypic switching. *Phenotypic switching* is an operational term indicating a change of phenotype (specifically, inducibility of steroidogenic enzyme genes) observed in individual cells and their descendants, of undetermined cause. Cell lines derived by transfection of SV40 T antigen exhibit a partial stabilization of expression of differentiated functions (see below).

When the induction of the steroid hydroxylases was tested over the early portion of the culture life span of bovine adrenocortical cells, it became apparent that the standard culture environment is not adequate for expression of all the differentiated function genes. Although there are pseudosubstrate effects that affect the activity of 11$\beta$-hydroxylase and 21-hydroxylase [7], the lack of expression of these enzymes in middle-passage cultures results from a defect in the accumulation of mRNA and not from a primary defect at the protein level [1]. At least in part, this may be corrected by the addition of the extracellular matrix preparation Matrigel [8] and by adding insulin-like growth factor I (IGF-I) as well as agents that increase intracellular cyclic AMP [9, 10]. When cells were plated in Matrigel, grown for 5 days, and then incubated with cholera toxin and IGF-I, Northern blotting showed that early- and middle-passage cells are able to express *CYP11B* (11$\beta$-hydroxylase) and *CYP21* (21-hydroxylase) [8] as well as *CYP17* (17$\alpha$-hydroxylase), *CYP11A* (cholesterol side-

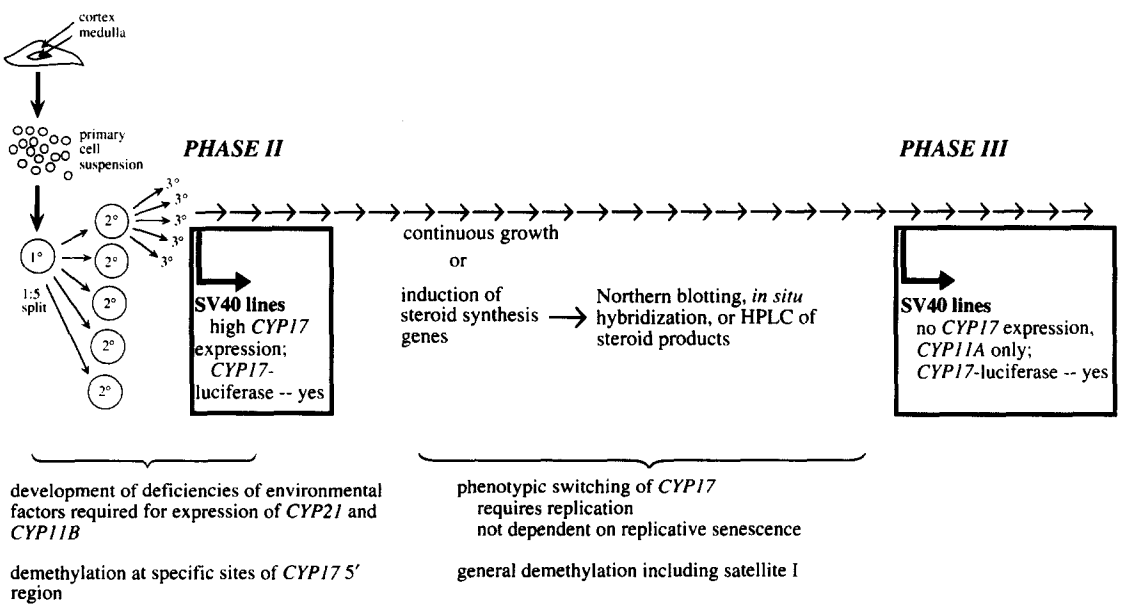
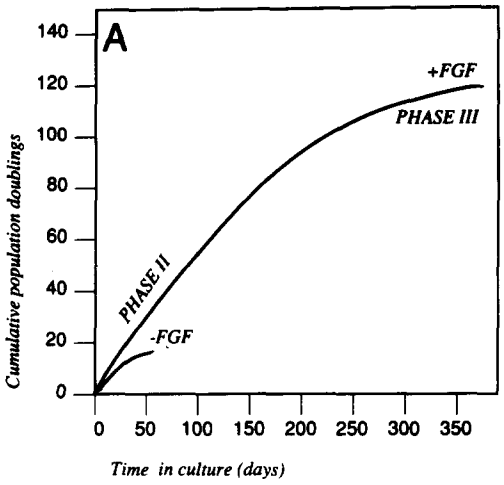
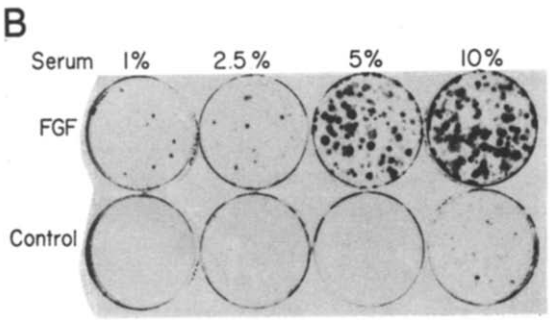



Fig. 1. Use of bovine adrenocortical cells in long-term culture to investigate cellular senescence mechanisms. Cells are isolated from the cortex, plated to form primary cultures, and after growth to confluence are subcultured to form secondary cultures, tertiary cultures, etc. with successive 1:5 splits. The line of arrows represents the continuous 1:5 subcultures of continuous growth of cells (*PHASE II*) until replicative senescence is reached (*PHASE III*). Cells may be allowed to grow continuously or the induction of steroidogenic enzyme genes may be investigated at different PDLs. The properties of SV40-transfected lines from early- and late-passages, and phases of the culture involving environmental deficiencies, phenotypic switching, and DNA methylation changes, are indicated.



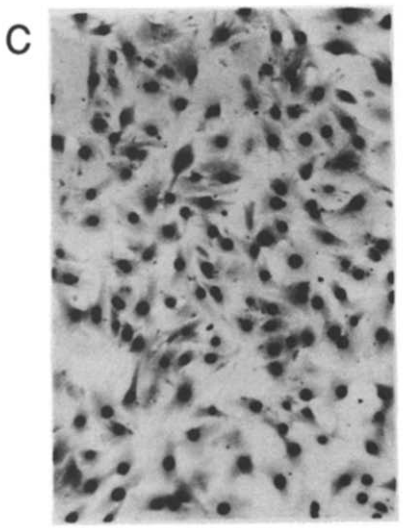




Fig. 2. Growth characteristics of bovine adrenocortical cells in culture. (A) Long-term growth in serum-containing medium is dependent on FGF. *PHASE II* and *PHASE III* refer to phases of the culture with exponential growth and cessation of growth, respectively. There is no "phase I" of growth out of an explant as occurs in fibroblast cultures. (B) Dependence of clonal growth on FGF and fetal bovine serum. (C) Primary culture of bovine adrenocortical cells labeled with [$^3$H]thymidine illustrating that almost all cells in the primary cell suspension enter the "phase II" of exponential growth. Data from Refs [4–6].

chain cleavage enzyme), and 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD). HPLC analysis of the products of conversion of 25-hydroxycholesterol confirmed that Matrigel allowed the restoration of a complete steroidogenic pathway to cortisol in these cultures.

## 2. PHENOTYPIC SWITCHING

In the second phase of the culture life span (after the appearance of environmental deficiencies) there is a decline in the expression of the genes of the later part of the steroidogenic pathway which is observed even under conditions that permit expression of a full steroidogenic pathway at earlier passages. Thus, at later passages, cholera toxin/IGF-I induces *CYP11A* and 3$\beta$-HSD but fails to induce *CYP11B*, *CYP21*, or *CYP17*, even when cells are grown in Matrigel [1, 2, 8]. We have investigated the decline in induction of *CYP17* specifically. Figure 3 shows that in mass cultures of bovine adrenocortical cells there is a general decline in the induced level of CYP17 mRNA and enzymatic activity over 80 to 100 population doublings.

Experiments using *in situ* hybridization show that this decline results predominantly from a decrease in the fraction of cells in the population capable of expression of *CYP17* (Fig. 3). Clones with high numbers of expressing cells can be used to derive subclones with a lower fraction of expressing cells or no expressing cells [3, 11]. Thus the loss of expression of *CYP17* results from phenotypic switching.

Investigation of the patterns of *CYP17* expression in colonies of cells gives insight into phenotypic switching. In Fig. 4, phenotypic switching was simulated by a simple computer model and compared with real colonies [13]. The model assumes that there is some probability of phenotypic switching occurring at each cell division; that colonies may have a division probability of 1.0 (all cells dividing) or $< 1.0$; and that in different colonies cells may have a greater or lesser capacity for migration between divisions. The model shows that real colonies can be simulated using values of probability of phenotypic switching of 3 to 6% per cell division.

## 3. METHYLATION OF THE *CYP17* GENES

Mechanisms must be sought for a high-frequency, presumably epigenetic, event that

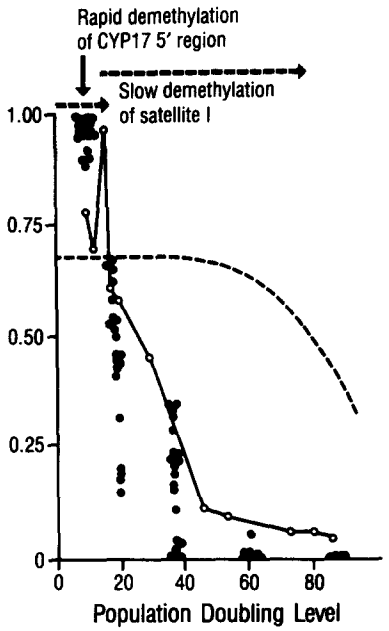



Fig. 3. Decline in expression of *CYP17* as a function of PDL in bovine adrenocortical cells and comparison with observed changes in DNA methylation (for details see text). Values on the ordinate are fractions of that in the primary culture. The solid line shows the decline in cholera toxin-induced levels of CYP17 mRNA [12]. Enzymatic activity shows a similar decrease [12]. The mRNA data are plotted together with data from hybridization of cells *in situ* to a *CYP17* cDNA probe [3]. Each point represents the percentage of cells hybridizing in small cholera toxin-treated clones produced from cells taken from mass cultures. The estimated PDL of the colony at the time of *CYP17* induction, rather than the PDL of the culture from which it was taken, is plotted. The growth rate of mass cultures, expressed as population doublings per day, is shown by the dashed line. Induction of *CYP17*, assessed either as the mRNA level in the culture as a whole, or as the number of cells capable of expression of the gene, shows a substantial decline before growth begins to slow down.

results in phenotypic switching. On a cell-by-cell basis phenotypic switching is not caused directly by replicative senescence [11]. We investigated the methylation of the *CYP17* genes, since extensive data show changes in methylation of DNA in aging *in vivo* and in cellular senescence [reviewed in 14]. We found a rapid demethylation occurring at two CpG sites in the 5′ region over the first few passages in culture well before phenotypic switching is observed [15]. Whether or not the demethylation is linked to the later loss of *CYP17* expression, its occurrence provides the opportunity for study of the regulation of synchronous changes in methylation. Presumably this *cis* event is regulated by intracellular *trans* events; thus there are presumably DNA sequence signals in the *CYP17* genes that target the region for demethylation.

In preliminary experiments, DNA was isolated from various cells and tissues, digested with *Eco*RI together with the methylation-sensitive restriction enzyme *Hpa*II or its non-methylation sensitive isoschizomer *Msp*I, blotted, and hybridized with a *CYP17* 5′ probe (−474/+29). The 4.7 kb *Eco*RI band was fully methylated in the adrenal cortex *in vivo* yet partially demethylated in other tissues and in adrenocortical cells in culture. This indicated that there are tissue-specific differences in methylation of the 5′ region of *CYP17* and that additional experiments should be performed to examine such differences. Probing with a *CYP17* cDNA probe showed that there are no differences in methylation in the coding region.

The data from these preliminary studies also indicated there are multiple *CYP17* genes in the bovine genome. A second *CYP17* gene was isolated from a bovine genomic library, subcloned, and sequenced to −2.5 kb. Evidence for a third gene is seen from Southern blots. In accordance with the current rules for the naming of the cytochrome *P*450 genes, we term these *CYP17A1*, *CYP17A2*, and *CYP17A3* (Fig. 5). *CYP17A1* corresponds to the previously isolated *CYP17* gene [16]. *CYP17A1* and *CYP17A2* are highly similar in sequence with conservation of the location of many restriction enzyme sites in the coding and 5′ regions of the genes. However, unique *Pae*R7I and *Xba*I sites at ~ −200 bp allow the identification of bands on Southern blots as resulting from *CYP17A1* or *CYP17A2*. Bands at 2.3 and 2.7 kb from the *Eco*RI site at the end of the first exon in *Eco*RI/*Hpa*II digests could be resolved as doublets, and the unique *Xba*I and *Pae*R7I sites were used to show that each band originates from a different gene.

When the methylation status of the 5′ region of *CYP17* was investigated in adrenocortical cells after being placed in culture, a rapid specific demethylation was observed at position H4 (see Fig. 5) in both *CYP17A1* and *CYP17A2* simultaneously, but not at neighboring sites (H1, H2, and H3). The demethylation was essentially complete by 6 passages (Fig. 6). In the intact adrenal cortex the 4.7 kb band from *CYP17A1* and *CYP17A2* is fully methylated. There is, however, a small band at 0.7 kb which apparently results from the existence of an additional *Msp*I/*Hpa*II site closer to the promoter in the third gene (*CYP17A3*). When this blot was reprobed with the cDNA the entire 8 kb coding region of the gene was shown to be fully methylated under all circumstances.

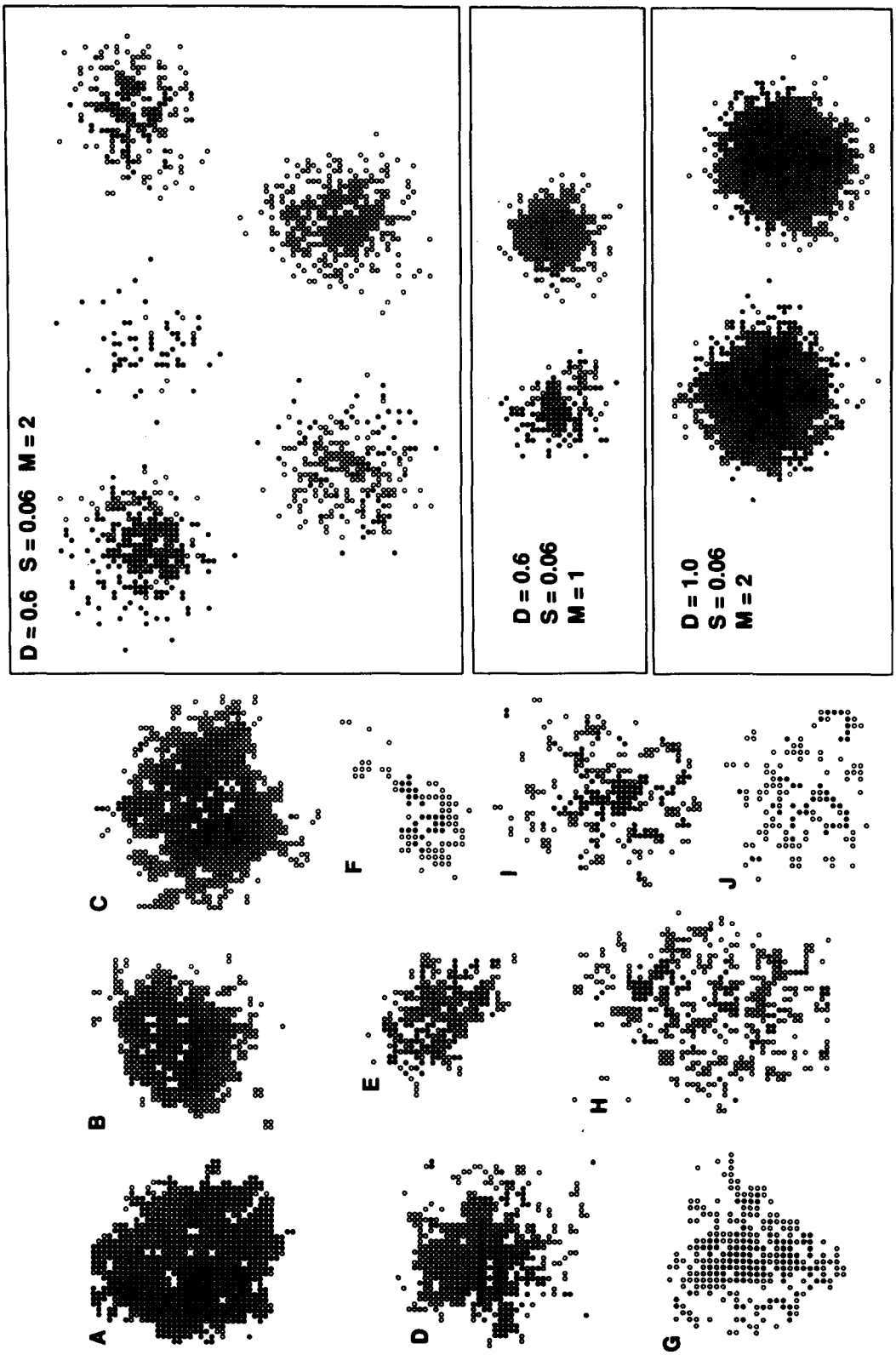



Fig. 4. Left panel: representations of actual colonies of cultured bovine adrenocortical cells showing expression of *CYP17* determined by *in situ* hybridization. Right panel: simulated colonies created using a computer program. In both cases, closed circles represent expressing cells and open circles represent non-expressing cells. For the digitization procedure and the simulation, see Ref. [13]. For the simulated colonies, values for parameters used are indicated as *D* (probability of division per time period), *S* (probability of phenotypic switching at each division), and *M* (number of migration steps per time period). All simulations are for 10 time periods. Upper panel: 5 successive runs of the simulation with the indicated values for the parameters are shown. Middle and lower panels: representative colonies produced by the program with the indicated values for parameters. From Ref. [13].

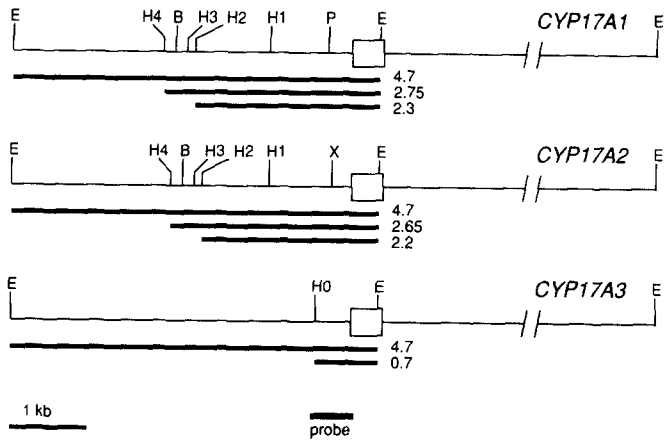



Fig. 5. Suggested maps of the bovine *CYP17* genes. In each gene, the box indicates the first exon; to the right is the 8.0 kb *Eco*RI fragment containing the rest of the coding region and to the left is the 4.7 kb *Eco*RI fragment of 5′ flanking region. E = *Eco*RI; H0–H4 = *Hpa*II/*Msp*I sites; P and X indicate unique *Pae*R7I/*Xho*I and *Xba*I sites, respectively; B = *Bsa*HI/*Acy*I. The position of the *Hpa*II/*Msp*I sites upstream of H0 was not determined in *CYP17A3*; a *Bsa*HI/*Acy*I site is present at approximately the same position as in *CYP17A2* [15]. The location of the −474/+29 probe used in Southern blots is indicated. The bars below each gene indicate the fragments, and their sizes in kb, that are detected by this probe when the gene is methylated, and when it is non-methylated at H4, H2, or H0. From Ref. [15].

Whereas H4 demethylates rapidly in early-passage adrenocortical cells, DNA from fibroblasts from three sources (adrenal cortex, liver, kidney) has a complete lack of methylation at position H2 in both *CYP17A1* and *CYP17A2* (Fig. 6). Results from intact liver and intact kidney show complex patterns presumably reflecting mixtures of different cells. In contrast, white blood cell DNA was always fully methylated in the *CYP17* genes. All cells and tissues

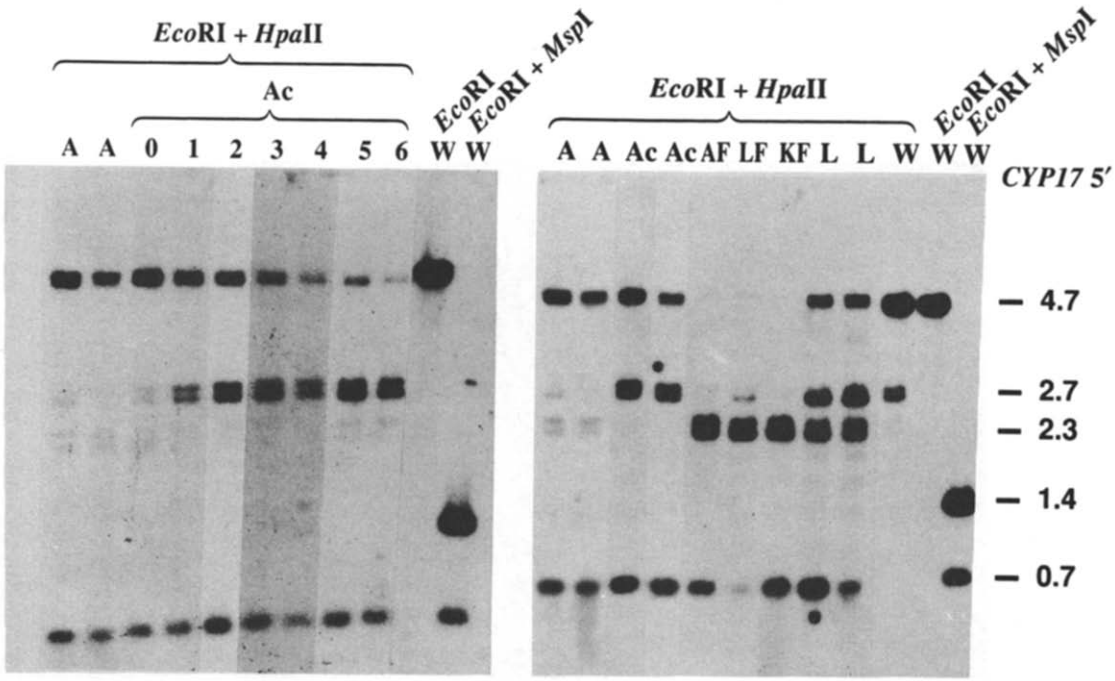



Fig. 6. Methylation of the 5′ region of *CYP17* in the adrenal cortex, cultured adrenocortical cells, and bovine tissues. DNA was isolated from the indicated cells and tissues, digested with *Eco*RI, *Msp*I, and/or *Hpa*II, blotted, and hybridized with *CYP17* 5′ probe. Abbreviations are as follows: (*Left*) A = DNA from bovine adrenal cortex (two different animals); Ac = cultures of adrenocortical cells. Numbers above lanes indicate passages of adrenocortical cell cultures which served as sources of DNA; 0 = primary culture. (*Right*) A = DNA from bovine adrenal cortex; Ac = cultured adrenocortical cells (two different primary cultures); AF, LF, and KF = fibroblast cultures grown from the adrenal cortex, liver, and kidney, respectively; L = intact liver; W = white blood cells. Numbers on the right indicate sizes in kb. From Ref. [15].

had completely methylated coding regions for the *CYP17* genes.

The enzyme *Bsa*HI (an isoschizomer of *Acy*I) cleaves a site between H3 and H4 (see Fig. 5) in a methylation-sensitive manner. This site was also found to be rapidly demethylated when cells were placed into culture. Moreover, this site was demethylated in all three *CYP17* genes both in fibroblasts and in cells cultured from the adrenal cortex.

These studies of methylation at sites H1 to H4 (*Hpa*II/*Msp*I sites) and the *Bsa*HI site show that there are tissue-specific differences in methylation in the 5′ region of the *CYP17* genes and that methylation of these sites changes as a function of cell division in cultured adrenocortical cells. *CYP17A1* and *CYP17A2* behave similarly with respect to changes in methylation supporting the concept that the genes respond in *trans* to changes in intracellular factors that regulate the methylation of their 5′ regions.

In contrast to changes in methylation in the 5′ region of the *CYP17* genes, which show a high degree of tissue and temporal specificity, later passage cells had more general changes in methylation, as typified by changes in methylation of satellite I. Satellite I is a 1.4 kb repeat which is normally highly methylated [17]. The presence of the 1.4 kb band in DNA digested with *Eco*RI, which cuts once per tandem repeat, shows that most copies of the satellite in most tissues are fully methylated. When adrenocortical cells are placed in culture there is a progressive demethylation of the satellite producing smaller bands. By population doubling level 55 very few copies of the satellite remain fully methylated. Most copies have complete demethylation of one or more sites, and the pattern begins to resemble that produced by *Msp*I. The significance of the satellite demethylation for proliferation and/or function of the cells is unknown.

#### 4. SV40 T ANTIGEN

Experiments with SV40 T antigen were performed to investigate the relationship between the decline in growth rate during senescence of bovine adrenocortical cells in culture and phenotypic switching [18]. We transfected both early- and late-passage cells with SV40 T antigen (plasmid pSV3neo). Early-passage clones showed high levels of expression of *CYP17* after induction with cyclic AMP, and maintained high levels of expression of *CYP17* through four successive recloning events, over a period of replication much longer than that achievable by non-transfected cells [18]. Expression of the other steroid hydroxylases in these clones also resembled that shown in the precursor cells before transfection. On regular culture dishes, SV40 T antigen-transfected clones of bovine adrenocortical cells behaved like middle-passage normal cells, i.e. *CYP17* was expressed but *CYP21* expression was low and *CYP11B* was not expressed. Like non-transfected cells, SV40 T antigen-transfected bovine adrenocortical cell clones grown in Matrigel and incubated with cholera toxin/IGF-I showed increased CYP17, CYP21, and CYP11B mRNA and synthesis of cortisol from 25-hydroxycholesterol precursor [8].

Thus we hypothesize that SV40 T antigen not only extends the replicative potential of bovine adrenocortical cells in culture but preserves the expression of *CYP17*, *CYP21*, and *CYP11B* as it was prior to transfection; either in the "on" state, when cells are transfected early in the culture life span, or in the "off" state, when senescent cells are transfected. Thus the introduction of SV40 T antigen in adrenocortical cells does not interfere with the proper expression of these genes, and also appears to stabilize their expression at a higher level, presumably by affecting phenotypic switching.

These data imply that SV40 T antigen is useful for the derivation of differentiated adrenocortical cell lines. We adapted the methods we had used for the production of SV40 T antigen-transfected bovine cells to human adrenocortical cells [2, 19, 20]. Like bovine adrenocortical cells and human fibroblasts, fetal human adrenocortical cells exhibit a finite "life span" in culture, but their total replicative potential is less than either (30–40 doublings total replicative capacity). Postnatal human adrenocortical cells in culture have a replicative potential strongly dependent on the age of the donor (Fig. 7). Cells from young donors have "life spans" of 20 to 40 doublings. Cells from donors of age 40 have some proliferative potential ($\sim$10 doublings), but in cultures from donors over 60 years of age, negligible cell division is observed. SV40 T antigen greatly extends the growth potential of fetal and postnatal human adrenocortical cells, as it does in bovine adrenocortical cells. Clones of fetal adrenocortical cells transfected with SV40 have a total replicative potential of between 60 and 80 population doublings, about twice that of the

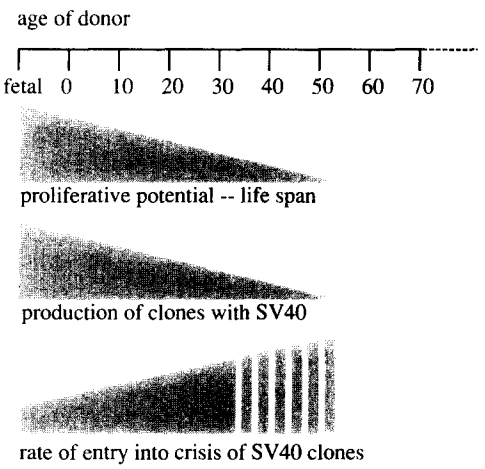



Fig. 7. Diagrammatic representation of the effects of donor age on proliferative potential, rate of production of clones after transfection with SV40, and rate of entry of such clones into "crisis". This diagram is based on data derived from cultured human fetal definitive zone adrenocortical cells, from zona fasciculata-reticularis cells from postnatal donors of 2, 11, and 18 years of age, and cells from one to three donors in each decade from age 20 to age 70. Data in part from Refs [2 and 22]; and C. Y. Cheng and P. J. Hornsby, unpublished.

non-transfected cells (Fig. 7). As expected from work on the effects of SV40 in human fibroblasts, these clones are not immortalized even though they have an extended replicative potential. After a period of proliferation that varies with the individual clone and is strongly dependent on the age of the donor, the cells enter "crisis". Cells from older adults infrequently gave rise to clones, which rapidly entered "crisis"; we have not observed the appearance of immortalized clones in "crisis" cultures. Similar results have been obtained with retroviral transfer of SV40 T antigen [21].

Functional studies on primary human adrenocortical cell cultures transferred into defined medium containing only bovine serum albumin, insulin or IGF-I, and ACTH, showed expression of a full differentiated steroidogenic pathway [23–25]. The replicative potential of fetal adrenocortical cells was sufficient to allow the production of clones [22]. These clones on testing under the same conditions as appropriate for primary cells proved to have a very low or negligible level of expression of steroidogenesis. However, as in bovine adrenocortical cells, SV40 T antigen expression resulted in maintenance of differentiated gene expression in fetal human adrenocortical cells [20]. None of the non-transfected fetal human adrenocortical cell clones studied had significant steroidogenesis, but SV40 T antigen-transfected fetal adrenocortical cell clones showed regulation of the steroidogenic pathway by cyclic AMP and protein kinase C similar to that of primary cultures of non-transfected cells [25]. In defined medium, cyclic AMP and IGF-I strongly increased CYP17 mRNA and 17$\alpha$-hydroxylase activity, assessed by conversion of progesterone or 25-hydroxy-[1,2-$^3$H]cholesterol. This induction was blocked by low concentrations of TPA (12-*O*-tetradecanoylphorbol-13-acetate) [20], as in non-transfected cells [25]. CYP11A mRNA and cholesterol side-chain cleavage activity were also greatly increased by cyclic AMP. 3$\beta$-HSD mRNA and activity were very low in control or with cyclic AMP alone, but were increased by the combination of cyclic AMP and TPA or serum. TPA enhanced 3$\beta$-HSD over a concentration range similar to that required to block induction of *CYP17*.

### 5. PROMOTER ACTIVITY OF *CYP17* WITH RESPECT TO PHENOTYPIC SWITCHING

In a second approach to the mechanism of phenotypic switching, we used luciferase reporter constructs to assess the activity of the *CYP17* promoter in bovine adrenocortical cells before and after phenotypic switching. We constructed two plasmids, pXP2-*CYP17* (−2544/+29) and PXP2-*CYP17* (−488/+29) from the 5′ region of *CYP17* linked to firefly luciferase cDNA. Cells at early-passage [population doubling level (PDL) 10] and late-passage (PDL 55) were cotransfected with either of these two plasmids ligated to pSV3neo and transfectants were selected in G418 [26].

The activity of the *CYP17* promoter in these transfectants was tested by growing cells in complete medium until semiconfluent and then transferring them into defined medium with cholera toxin and IGF-I for 6 h. As shown in Fig. 8, induced luciferase activity was actually higher in late- than in early-passage cells, at least for the −488 construct. The lower response of the −2544 construct in cells from both PDLs indicates the potential existence of a negative regulatory element. Sequence analysis indicates that the region between −300 and −1800 consists of several repeats that are common in bovine genes, whereas the region that undergoes demethylation does not [15]. Expression of the endogenous *CYP17* gene corresponds to what would be expected from non-transfected cells; at PDL 10 the gene is well expressed but not at PDL 55. These data indicate that the loss

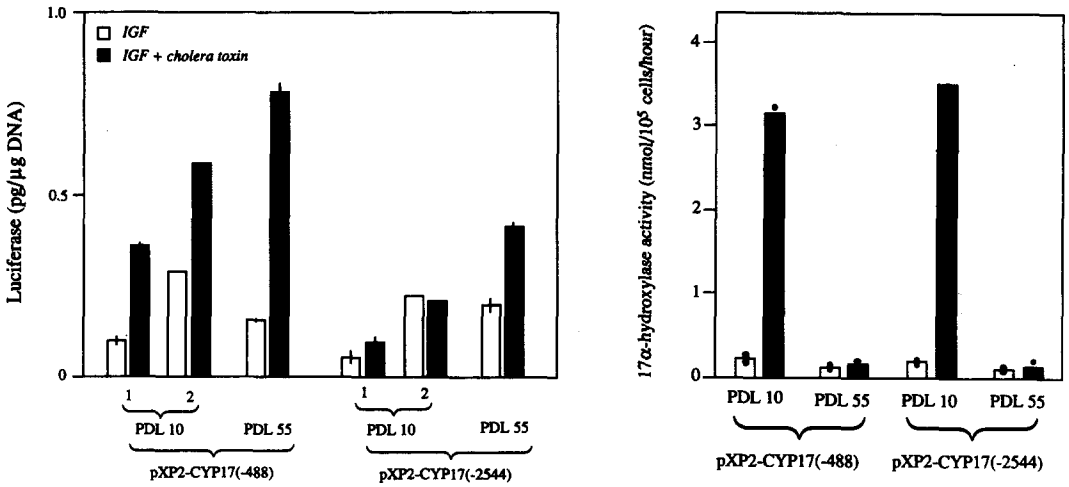



Fig. 8. Promoter activity of fusion genes containing different lengths of the 5′ flanking region of *CYP17* linked to luciferase in bovine adrenocortical cells before and after loss of endogenous *CYP17* expression by phenotypic switching. (*Left*) Luciferase levels in defined medium (IGF-I added) or defined medium with IGF-I and cholera toxin in bovine adrenocortical cells stably cotransfected with pSV3neo and luciferase constructs (pXP2; see Ref. [27]) with −488/+29 or −2544/+29 of *CYP17* 5′ region. Cells at PDL 10 and 55 were transfected. Two separate transfections (indicated as "1" and "2") to yield two separate pools of clones were performed with the −488/+29 plasmid. (*Right*) 17α-Hydroxylase activity under the same conditions in the different transfected cell populations. From Ref. [26].

of cyclic AMP-inducible *CYP17* expression during senescence is not due to a loss in positive regulatory factors that act within −2544 to +29.

Because of technical difficulties with transient transfection of late-passage bovine adrenocortical cells, these experiments were performed using stable transfection and pools of clones. Luciferase activity was consistently induced by cholera toxin/IGF-I over 5 passages in pooled clones derived by transfection of early-passage cells with the −488 construct. Another experiment was done to make sure that the lower expression of the −2544 construct versus the −488 construct was reflected in individual clones of cells as well as pools. There was consistent cholera toxin/IGF-I induction of luciferase activity in individual clones derived by transfection of early-passage cells with the −488 construct and relatively weak or no induction in clones with the −2544 construct [26].

## 6. SUMMARY

These experiments permit the following conclusions.

Bovine adrenocortical cells undergo a process in which expression of steroid hydroxylases is lost progressively as a function of PDL in culture. When the different environmental factors required for induction of *CYP11B* and *CYP21* are taken into account, induction of these genes disappears with the same kinetics as previously shown for *CYP17* as a function of PDL. Phenotypic switching was observed specifically for *CYP17* but may simultaneously affect the expression of *CYP21* and *CYP11B*. Further experiments will be required to examine the possibility that a factor common to the expression of all three genes changes with respect to PDL in bovine adrenocortical cells.

The mechanism of phenotypic switching is unknown. The process may be affected by SV40 T antigen. The specific demethylation of the 5′ region of the *CYP17* genes suggests that this event should be studied for its possible consequences. However, data from reporter constructs suggest that phenotypic switching does not result from a simple loss of regulatory factors that act within 2.5 kb of the promoter.

Further study of the characteristics of the behavior of the steroid hydroxylase genes during senescence of adrenocortical cells in culture will likely yield additional valuable data concerning basic cell biological questions of the long-term relationship of such changes to aging as well as elucidating new physiological mechanisms for the regulation of these genes.

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