

Construction of a Leydig cell line synthesizing testosterone under gonadotropin stimulation: A complex endocrine function immortalized by cell hybridization

(clonal cell lines/steroidogenesis)

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ABSTRACT Hybridization between a mouse Leydig tumor cell line, MA-10, which produces cyclic AMP and progesterone under human chorionic gonadotropin (hCG) stimulation, and freshly isolated mouse Leydig cells gave rise to 54 hybrid clones, one of which, LK17, was capable of hCG-stimulated testosterone production. Subcloning of this hybrid resulted in the emergence of a subclone, K9, whose testosterone production is more than 10 times that of parent clone LK17, after hCG stimulation, with an ED₅₀ of 37 pM. Testosterone synthesis by K9 cells was multiplied by 25 after gonadotropin stimulation, and binding of hCG declined after prolonged exposure to the hormone. These similarities with murine Leydig cells in primary culture make the K9 clone an attractive alternative for physiological studies.

Production of testosterone by Leydig cells is the outcome of a complex steroidogenic pathway, initiated by the cleavage of the side chain of cholesterol. This step is triggered by the binding of luteinizing gonadotropin to luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptors located on the cell plasma membrane, followed by the activation of adenylate cyclase and protein kinase (see ref. 1 for review). Leydig cells in primary culture have been used to study the mechanism of action of gonadotropin, but testosterone production is not sustained more than a few days (2-6). Clonal cell lines derived from mouse (7-9) and rat (10) Leydig cell tumors, although they may retain LH/hCG receptors, are unable to pursue steroidogenesis beyond progesterone synthesis. Using somatic cell hybridization between the MA-10 mouse Leydig tumor cell line obtained by Ascoli (11) and freshly isolated mouse Leydig cells, we have immortalized the full steroidogenic function of the latter. The strategy used for obtaining this testosterone-producing gonadotropin-sensitive permanent cell line is described below.

MATERIALS AND METHODS

Parental Cells. A clonal mouse Leydig tumor cell line, MA-10 (11), was kindly provided by Mario Ascoli (Nashville, TN). This cell line responds to hCG stimulation by an increased secretion of progesterone and 20 α -dehydroprogesterone. It was made hypoxanthine/guanine phosphoribosyltransferase negative (HGPRT⁻) by mutation using ethyl methanesulfonate, added at 300 μ g/ml for 14 hr to cells grown to confluence in Dulbecco's modified Eagle's medium containing 15% fetal calf serum. The treated cells were allowed to recover 7 days in the initial medium, and HGPRT⁻ cells were then selected by addition of 100 μ M 6-thioguanine. Mutant colonies appeared 3 weeks later. A mutant clone, LK, was chosen for its endocrinological similarity to the

MA-10 parent cell line—namely, the ability to respond to hCG stimulation by an increased progesterone production.

Fresh Leydig cells, isolated from the testes of 6-week-old BALB/c mice, were purified and maintained in primary culture as previously described (6, 12). Ouabain at 20 μ M will kill fresh Leydig cells but not the LK clone.

Cell Hybridization and Subcloning. Twelve hours after plating of the fresh Leydig cells, 10⁵ of these were mixed with LK cells at a 1:1 ratio and submitted to electrofusion as previously described (13). Fifty-four hybrids were selected by culture in the presence of 100 μ M hypoxanthine/0.4 μ M aminopterin/16 μ M thymidine (HAT medium) as well as 20 μ M ouabain. The latter was omitted after four subcultures, but HAT selection was maintained throughout the culture period. Subcloning was effected by limiting dilution in conditions yielding 20% wells with viable cells.

Testing of Hybrid Cell Clones. Prior to biochemical testing, the cells were incubated 7 days in a serum-free chemically defined medium (6). Binding of highly purified hCG (CR 123, 12,750 international units/mg, prepared by R. E. Canfield) was studied as described (6) after iodination of the hormone by the lactoperoxidase method (14) to a specific activity of 50-60 μ Ci/ μ g (1 Ci = 37 GBq).

Cyclic AMP (cAMP) production was measured in the presence or absence of 1 nM hCG after incubation of cells in 0.5 ml of medium containing 0.2 mM 3-isobutyl-1-methylxanthine for 1 hr. The incubation medium was added to 4 ml of cold ethanol, and the material was evaporated to dryness, acetylated, and submitted to radioimmunoassay, according to Steiner *et al.* (15). Progesterone and testosterone production in the presence of 0-1.3 nM hCG were measured by radioimmunoassay as described (16). For screening of hybrid clones, results are expressed per 10⁵ cells counted after trypsinization. Results of the study of LK17 subclones are expressed per mg of protein, measured by the method of Lowry *et al.* (17) after dissolving the cell monolayer in 0.4% sodium deoxycholate. Results are presented as mean \pm SD, and statistical evaluation of the effectiveness of hCG stimulation was performed by Student's *t* test.

RESULTS

Screening of Fusion Products and Subclones. Nucleotide and hormone production of the primary hybrids was investigated after six subcultures at the latest. Basal secretion of cAMP and progesterone was extremely variable, ranging between 0.73 \pm 0.05 and 3.53 \pm 0.15 pmol per 10⁵ cells per hr for cAMP and between 0.11 \pm 0.02 and 9.6 \pm 1.1 ng per

Abbreviations: LH, luteinizing hormone (lutropin); hCG, human chorionic gonadotropin (choriogonadotropin); HGPRT, hypoxanthine/guanine phosphoribosyltransferase.

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10^5 cells per 4 hr for progesterone. The hCG-stimulated production of cAMP, progesterone, and testosterone by primary hybrid clones is shown in Fig. 1. Productions of the three factors by a given clone were not correlated. Whereas a majority of clones responded to hCG stimulation by an increased cAMP or progesterone output, only one, LK17,

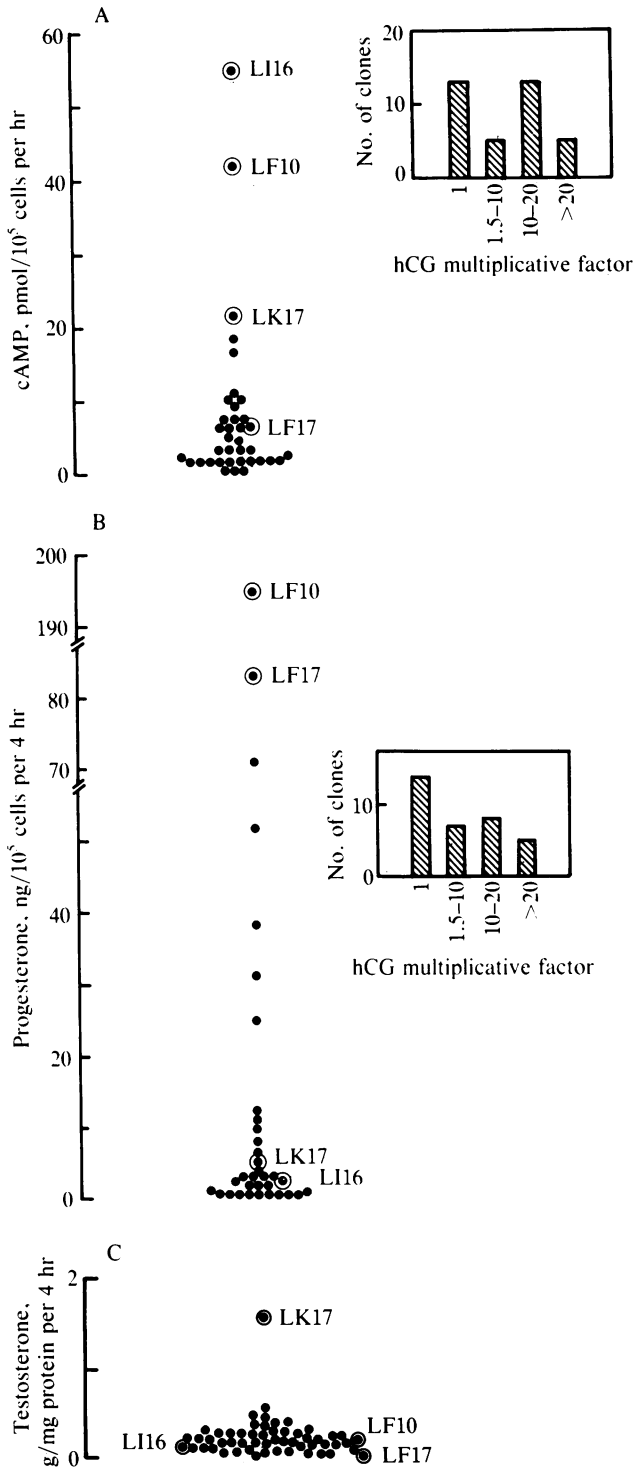


FIG. 1. Production of cAMP (A), progesterone (B), and testosterone (C) by primary hybrids. Cells were incubated for 1 hr (A) or 4 hr (B and C) in the presence of 1 nM hCG. The radioimmunoassay values shown are the mean of three experiments; SDs were less than 10%. LI16, LF10, LK17, and LF17: hybrid clones producing high amounts of cAMP, progesterone, or testosterone. (Insets) Distribution of the hybrids as a function of their hCG multiplicative factor, which expresses the relationship between basal and hCG-stimulated production of cAMP or progesterone.

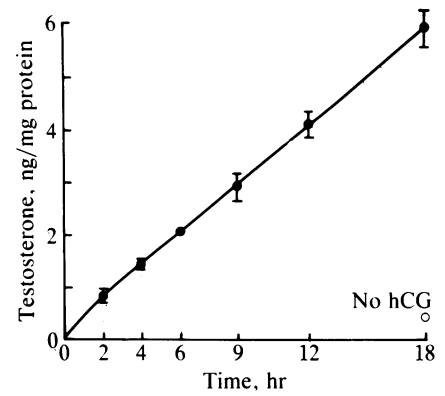


FIG. 2. Kinetics of testosterone production by primary hybrid LK17 in the presence of 1 nM hCG. The value obtained in the absence of hCG is shown by \circ . Values shown are the mean and SD of three determinations. Testosterone production by LK17 was linear for 18 hr.

was capable of hCG-stimulated testosterone production (Fig. 1C), which was linear as a function of time (Fig. 2). Subcloning of LK17 yielded 74 clones, 4 of which produced more than 10 times the amount of testosterone synthesized by their LK17 parent (Fig. 3). Subclone K9 was selected on the basis of its superior growth velocity. Its doubling time is 18 hr in medium supplemented by 15% fetal calf serum.

The relationship between basal and hCG-stimulated production (hCG multiplicative factor) is given in the histograms

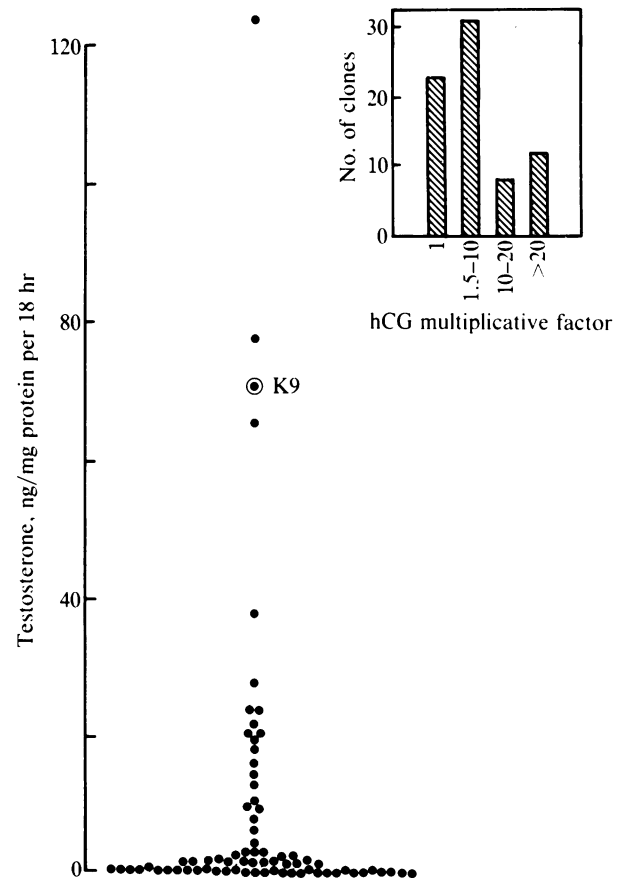


FIG. 3. Production of testosterone by subclones of LK17, incubated 18 hr in the presence of 1 nM hCG. The radioimmunoassay values shown are the mean of three experiments; SDs were less than 10%. (Inset) Distribution of the subclones as a function of their hCG multiplicative factor, which expresses the relationship between basal and hCG-stimulated testosterone production.

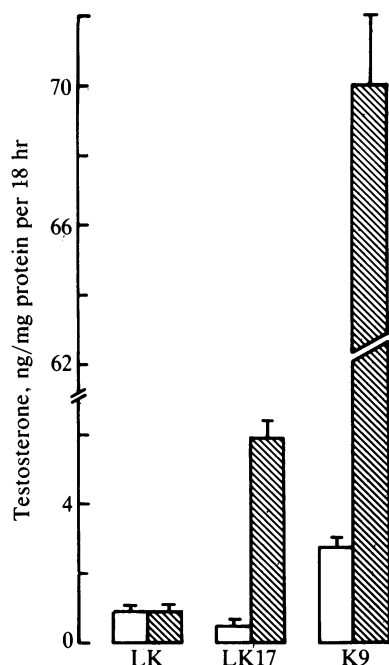


FIG. 4. Basal (empty bars) and hCG-stimulated (hatched bars) testosterone production of hybrid clone LK17, its LK parent, and its K9 subclone. Cells were stimulated for 18 hr by 1 nM hCG. Parent clone LK produces minimal amounts of testosterone, even in the presence of hCG. The testosterone production of hybrid clone LK17 is increased 10-fold by hCG stimulation. Basal and hCG-stimulated testosterone synthesis by subclone K9 is dramatically enhanced.

in Figs. 1 and 3. The multiplicative factor was not related to the absolute level of hCG-stimulated production by a given clone (results not shown).

Characterization of Subclone K9. Basal and hCG-stimulated testosterone production of LK17, its parent clone LK, and its subclone K9 is shown in Fig. 4. Clone LK produces minimal amounts of testosterone, even under hCG stimulation. Clone LK17 synthesizes 5.85 ± 0.55 ng of testosterone per mg of protein per 18 hr when stimulated by hCG, representing a multiplicative factor of 10. The performances of subclone K9 are even better: under hCG stimulation, it produces 71 ± 2.12 ng of testosterone per mg of protein per 18 hr, and the multiplicative factor reaches a value of 25. Clone K9 is highly sensitive to hCG stimulation (Fig. 5): a 50% stimulation of testosterone production is obtained with 37 pM hCG. The kinetics of binding of iodinated hCG to K9

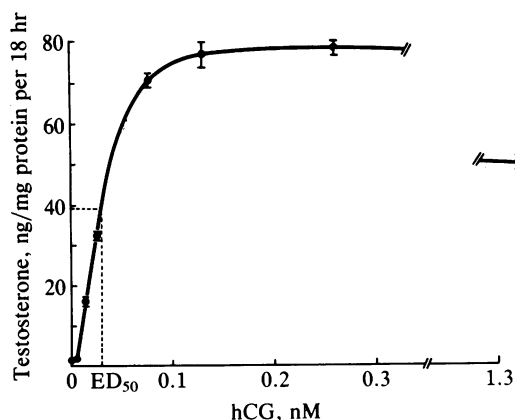


FIG. 5. Dose-response curve of hCG-stimulated testosterone production by subclone K9. Values shown are the mean and SD of quadruplicate determinations. ED₅₀ is 37 pM hCG.

cells are shown on Fig. 6. A maximum (314 pg per 10^5 cells) is reached by 2 hr, then binding declines. At 24 hr, approximately 15% of radioactivity is still bound to the cells.

DISCUSSION

Culture of purified cell populations in defined media is often preferred to *in vivo* studies for the investigation of specialized cell functions and their regulation. Unfortunately, cells in primary culture rapidly undergo dedifferentiation: for instance, Leydig cells cease to synthesize testosterone after a few days (5, 6, 18–20). Construction of permanent cell lines expressing the function of interest is an attractive alternative to the tedious repetition of cell isolation. Several methods have been applied to this goal. Introduction of a cloned gene into eukaryotic cells has allowed stable expression of human immunoglobulin (21), avian β -globin (22), growth hormone (23), preprorenin (24), and anti-Müllerian hormone (25). However, transfected cells can only express the product of a single inserted gene and cannot support a complex metabolic pathway, such as the one required for the biosynthesis of testosterone. Permanent cell lines can also be generated from tumor or transformed cells, but partial or complete dedifferentiation is a frequent corollary of transformation. The functional activity of transformed cell lines is usually limited to the expression of a single protein, such as human choriongonadotropin (26), insulin (27), or renin (28). If, however, many enzymes or proteins are required for the biosynthesis of the desired product, this strategy is usually not successful. For instance, the Leydig tumor cell lines described by Shin (7), Rebois (9), and Ascoli (11) do not produce testosterone, being unable to carry out steroidogenesis beyond the progesterone step.

Hybridization between a transformed cell line from a tissue originally expressing the desired function and fresh cells isolated from the same tissue in the same species has won the Nobel prize for Köhler and Milstein (29), who applied this strategy to the production of monoclonal antibodies. The hybrids inherit capacity for unlimited growth from their transformed parent and the capacity to express facultative traits from the freshly prepared cells. This method has been successfully applied to production of human factor VIII-related antigen (30) and glucagon (31). Furthermore, intraspecific intratissue cell hybridization is particularly well suited to immortalization of complex enzymatic pathways. Thus, cell hybrids derived from hepatocytes produce several varieties of bile acids (32). Other hybrid cell lines, derived from granulosa cells, synthesize

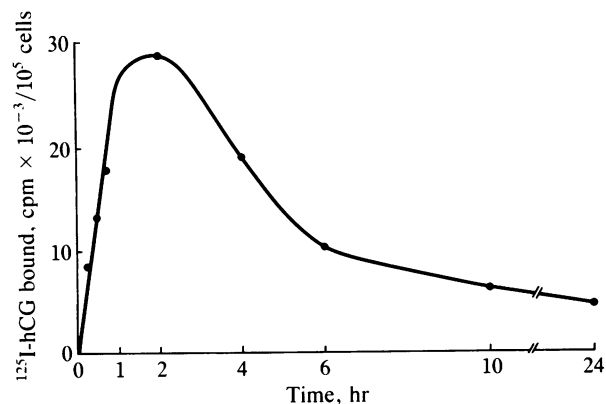


FIG. 6. Kinetics of hCG binding to K9 cells. Cells were incubated in the presence of iodinated hCG (20 μ g/ml, specific activity 50–60 μ Ci/ μ g) and radioactivity was measured after thorough rinsing, at the indicated time intervals. Binding is maximal at 2 hr and decreases thereafter. The values represent the average of two independent experiments performed in quadruplicate.

progesterone (33). Continuation of steroidogenesis beyond this early step was, however, not reported, and the hybrids did not respond to the hormones that regulate steroid production of normal granulosa cells.

Using hybridization between fresh mouse Leydig cells and a clonal cell line derived from a tumor of mouse Leydig cells, we have constructed a hybrid cell line secreting testosterone under hCG stimulation. Although the hCG-stimulated testosterone production of our K9 cell line was significantly lower than that usually reported for fresh rodent Leydig cells, the hCG sensitivity was comparable, as shown by the values of ED₅₀ (5, 6, 34, 35). A further resemblance to normal Leydig cells can be found in the progressive decrease of hCG binding after prolonged stimulation (Fig. 6). Since, according to Ketelslegers *et al.* (36), degradation of iodinated hCG is minimal during the first 6 hr of incubation, the decrease of hCG binding observed by us from 3 to 6 hr is probably mostly related to the down-regulation of receptors (37). The peak amount of hCG bound to K9 cells after 2 hr incubation significantly exceeded that binding to rodent Leydig cells in primary culture (5, 6, 35).

Production of testosterone, the terminal product of steroidogenesis in Leydig cells, requires the presence of five specific enzymatic activities—namely, cholesterol side-chain cleavage cytochrome P-450, 3 β -hydroxysteroid dehydrogenase, 17-hydroxylase, 17-20 lyase, and 17-cetoreductase. To respond to hCG stimulation, cells must also be equipped with functional receptors for the hormone, adenylate cyclase, and protein kinases. The multiplicity of the proteins necessary for the completion and regulation of steroidogenesis probably explains why it has been impossible to obtain this result by using other methods of construction of clonal cell lines, and why only a single hybrid out of 54 met the stipulated requirements (Fig. 1C). Subcloning of this hybrid dramatically improved its efficiency (Fig. 4). The fall of testosterone production observed after 2–3 months, which necessitates routine subcloning, could be due to overgrowth of cultures by cells having reverted to the phenotype of the MA-10 parent. All features except testosterone production were maintained after a 6-month culture period. Cells frozen before the 10th passage secreted testosterone after thawing.

Dedifferentiation, even beyond the level reached by the transformed parent, is the general rule in hybrids derived from cells of different tissues even when they are related, as are adrenal and Leydig cells (38). In the present case, many hybrids had even lost the capacity to synthesize cAMP and progesterone, which are produced by their MA-10 parent, but the fact that both parents were of Leydig cell origin probably allowed some hybrids to escape extinction of their facultative traits. The K9 hybrid cell line we describe represents a convenient and economical supply of homogeneous material for the study of Leydig cell function and regulation.

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