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Transgenic mice expressing the tumor marker germ cell alkaline phosphatase: An *in vivo* tumor model for human cancer antigens

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ABSTRACT **We have generated a series of transgenic mouse lines harboring the entire human germ cell alkaline phosphatase (GCAP) gene linked to progressively longer sequences of flanking DNA. A 450-bp promoter sequence directs the expression of GCAP to the intestine and endothelial cells, while a 5′ sequence of 1.7 kb directs GCAP expression to the spermatogenic lineage and to the eight-cell through the blastocyst stage of preimplantation development. The expression of GCAP in these FVB/N transgenic mice induces a cellular immune tolerance to GCAP. When mouse fibrosarcoma MO4 cells (C3H derived), stably transfected with the cloned GCAP gene, were injected s.c. in nontransgenic control (C3H× FVB/N) hybrid mice, GCAP-positive tumor cells were rejected. However, when GCAP-expressing transgenic (C3H×FVB/N) hybrid mice were challenged with these cells, GCAP-positive tumors developed. Tumors also developed in the transgenic hybrid mice upon injection of MO4 cells transfected with the highly homologous placental alkaline phosphatase (PLAP) cDNA in spite of the presence in PLAP of 10 amino acids that are different from the corresponding residues in GCAP. These GCAP transgenic mice will allow the study of the immune response associated with the repeated administration of conjugated or derivatized anti-GCAP and anti-PLAP monoclonal antibodies. They will also enable evaluation of the therapeutic potential of bifunctional antibodies for T-cell recruitment and destruction of GCAP/PLAP-producing tumor cells.**

Human placental (PLAP) and germ cell (GCAP) alkaline phosphatase are oncodevelopmental isozymes displaying 98% sequence similarity (1, 2). PLAP is expressed in the syncytiotrophoblast from the 12th week of pregnancy to term (3) and is often reexpressed ectopically in cancer of the lung, breast, cervix, and ovaries (4). GCAP is expressed in migrating primordial germ cells and gonocytes (5, 6) and is upregulated in carcinoma *in situ* (7) and in seminoma of the testis (8). Serological evaluation of PLAP and GCAP provides useful clinical information in management of these patients (9, 10). Because of their accessible membranous location, tumoral PLAP and GCAP have been targeted during radioimmunoscintigraphy studies in nude mice (11, 12) and in patients (13). Encouraging immunotherapeutic results have been obtained *in vitro* with ricin A-conjugated anti-PLAP monoclonal antibodies (14) and *in vivo* after tumor treatment with ^{131}I-labeled antibodies in nude mice (15). Although the radioimmunotherapeutic treatment of tumors can be studied in nude mice, these animals are immunodeficient and do not allow any evaluation of T-cell-dependent immunological responses during immunotherapy. In this report, we show that mice, transgenic for the human GCAP gene, display cellular immune tolerance to syngeneic tumor cells that are transfected with the GCAP or the PLAP gene. Consequently, an *in vivo* murine cancer model is established that not only allows the study of cellular immune responses during tumor immunotherapy but is also suited to study the therapeutic potential of immunological T-cell recruitment for the destruction of GCAP/PLAP-producing tumor cells.

MATERIALS AND METHODS

Generation of Transgenic Mice. The GCAP constructs used for oocyte injection (see Fig. 1) were assembled in the KS+ Bluescript (Stratagene) vector from cloned DNA subfragments used to sequence the GCAP gene (2). A 4.5-kb *Eco*RI/*Hin*dIII fragment (construction I), a 5.7-kb *Not* I/*Hin*dIII fragment (construction II), an 8.5-kb *Not* I/*Sal* I fragment (construction III), an 11.8-kb *Xba* I/*Sal* I fragment (construction IV), and a 17.0-kb *Sal* I fragment (construction V) were purified and injected into fertilized oocytes according to established protocols (16). CB6 F_1 female mice were used as oocyte donors for DNA construction I and FVB/N female mice were used as oocyte donors for constructions II, III, IV, and V. The viable injected eggs were reimplanted in both oviducts of recipient females (CD1) at 20–25 oocytes per female and pregnancies were allowed to continue to term. Tail DNA was extracted as described (16) and was used in Southern blots (17) to examine integration of the transgene. Copy number was determined by analysis of duplicate Southern blots of *Bam*HI-digested genomic DNA hybridized with ^{32}P-labeled 2.0-kb *Eco*RI/*Kpn* I fragment of the PLAP cDNA (1) to detect the number of integrated GCAP gene copies or with a 550-bp cDNA fragment of the endogenous mouse EAP gene (18) to correct for small differences in the amount of loaded DNA. Human genomic DNA was used as standard sample with two copies of GCAP gene. The radioactive signal on the filters was measured with an Ambis radioanalytic system (AMBIS Systems, San Diego).

GCAP Expression. Mouse tissues were fixed in 10% phosphate-buffered formalin and prepared for microtome sectioning as described (18). Tumors were excised and fixed for 1.5 h at room temperature in 4% formalin, buffered in 0.1 M sodium cacodylate (pH 7.4), and embedded in paraffin for sectioning. Immunohistochemical identification of GCAP was performed as described (18) except that affinity-purified rabbit anti-PLAP antibody (12.5 μg/ml) was incubated as first antibody for 60 min at room temperature. Enzyme antigen immunoassay for GCAP was performed as described (19). Reverse transcriptase (RT)-PCR was performed following reaction conditions recently described (18) using as 5′ primer the sequence 5′-CTG AGT ACC CAG ATG ACT ACA GCC AAG G-3′ (identical to nt 1871–1897), which anneals within exon VI, and using as 3′ primer the sequence

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Abbreviations: GCAP, germ cell alkaline phosphatase; PLAP, placental alkaline phosphatase; FACS, fluorescence-activated cell sorting; RT, reverse transcriptase.

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5′-GAG CCC CGA GTA TCG GCA GCA GTC AGC A-3′ (reverse complement of nt 3121–3148), which anneals within exon XI of the GCAP gene (2).

MO4 Tumor Cell Transfection, Fluorescence-Activated Cell Sorting (FACS) Analysis, and Tumor Growth. The MO4 GCAP transfectant was produced by cotransfection with the pSVT7-GCAP vector and pSV2-neo as described (20), upon which positive cells were selected with geneticin at 1 mg/ml. The MO4 PLAP transfectants were previously reported (12, 21). The membrane PLAP and GCAP content of the PLAP or GCAP transfected cells was investigated by FACS. Cells were resuspended in phosphate-buffered saline (PBS) containing 5% fetal calf serum and 0.04% NaN_3 and divided at 10^5 cells per tube. Upon reaction at 0°C for 30 min with the anti-PLAP monoclonal antibody 7E8 at 5 μg/ml (20), cells were washed with PBS and subjected to a second incubation step with fluorescein isothiocyanate-labeled rabbit anti-mouse $F(ab')_2$ fragment IgG (Dakopatts, Glostrup, Denmark) for 30 min. A homogeneous GCAP-transfected MO4 cell population was obtained through subcloning by limited dilution, and a homogeneous PLAP-transfected MO4 cell population was selected by FACS in an attempt to select a cellular population having an antigen density comparable to that of the selected GCAP transfected clone.

Control (untransfected) and transfected MO4 (C3H derived) cells were suspended in saline at 10^7 cells per ml and $\approx 10^6$ cells were injected s.c. in the left (control cells) and right (transfected cells) thigh of C3H mice and of F_1 and F_2 (C3H×FVB/N) hybrid mice. Seven days after the first detection of a palpable mass, tumor volumes were measured.

RESULTS

Generation of Transgenic Mice. To obtain transgenic mice displaying different patterns of expression of the GCAP gene, five DNA constructions were used for injection into oocytes (Fig. 1). Construction I contained a minimal promoter region (450 bp), including a TATA box and an Sp1 binding site (2), linked to the entire GCAP structural gene (4 kb long). Three founders were obtained from 84 live pups (3f/84p). Construction II (5.7 kb) contained 1.7 kb of promoter sequences but was otherwise identical to construction I (4f/44p). Construction III (8.5 kb) was identical to construction II but was extended at the 3′ end by an additional 2.8-kb sequence derived from the GCAP gene (9f/94p). Construction IV (11.8 kb) was identical to construction III but contained a 3.3-kb spacer DNA fragment, derived from phage λ, upstream from the 1.7-kb promoter sequence (17f/72p). Construction V (17.0 kb) was identical to construction III but contained 8.5 kb of spacer λ DNA upstream from the 1.7-kb promoter sequence (3f/115p). The spacer λ DNA was included in constructions IV and V to provide better isolation of the 5′ regulatory regions of the GCAP gene from the neighboring DNA at the integration sites. Southern blot analysis of the offspring tail DNA indicated that in 16 of 20 analyzed founder pedigrees transgenic for the different constructions (of the total 36 founders) the transgene was inherited in a simple Mendelian fashion, while copy number varied from 1 to >100. In particular, founders P2, P29, and P94 from construction V integrated the transgene at a single chromosomal site, as evidenced by breeding and Southern blot analysis, and inherited the transgene in a simple Mendelian fashion with copy numbers of 8, 11, and 24, respectively (data not shown).

Expression of GCAP in Transgenic Mice. All founder mice were bred and the first generation was screened for expression of the GCAP transgene. Immunohistochemical screening of GCAP expression in two pedigrees of construction I revealed high GCAP expression in the intestinal epithelium (Fig. 2*A*) and in endothelial cells (Fig. 2*B*). The expression in endothelial cells was present throughout the body, including the testis, in fetal, neonatal, and adult life, while intestinal expression was only observable at the neonatal and adult stages. Enzyme antigen immunoassays revealed levels of 14.3 ± 8.2 ng of circulating GCAP antigen per ml in the serum of construction I transgenic mice.

Immunohistochemical analyses of offspring from the founders derived from DNA constructions II, III, and IV failed to detect any appreciable amount of antigen in any tissue. However, all three constructions expressed GCAP mRNA exclusively in the testis of the transgenic mice as detected by RT-PCR (Fig. 3). In contrast, when we analyzed construction V, we found testis-specific expression of both GCAP mRNA and GCAP antigen in the testis of offspring from all three founders (Fig. 2). The level and tissue distribution of expression, based on immunohistochemical staining and enzyme antigen immunoassay on tissue extracts, is comparable in the P2, P29, and P94 families. Immunohistochemical stainings (Fig. 2 *C–G*) indicate that germ cells at the stage of spermatocytes and spermatids are positive for human GCAP. Immunogold staining and higher-resolution electron microscopy of the tissues confirmed these results (data not shown). Spermatozoa found in the caput region of the epididymis are positive for GCAP, while those found in the corpus and cauda regions are negative. All other adult mouse tissues are negative as are serum levels of GCAP. The GCAP mRNA is also detected at the eight-cell stage and at the blastocyst stage preimplantation embryo (Fig. 3), stages in which the endogenous mouse embryonic alkaline phosphatase isozyme gene is expressed (23).

Development of GCAP-Positive Tumors in Transgenic Mice. Mouse fibrosarcoma MO4 cells produce invasive tumors in syngeneic C3H mice (24). These cells were stably transfected with the GCAP and the PLAP gene, respectively, and se-

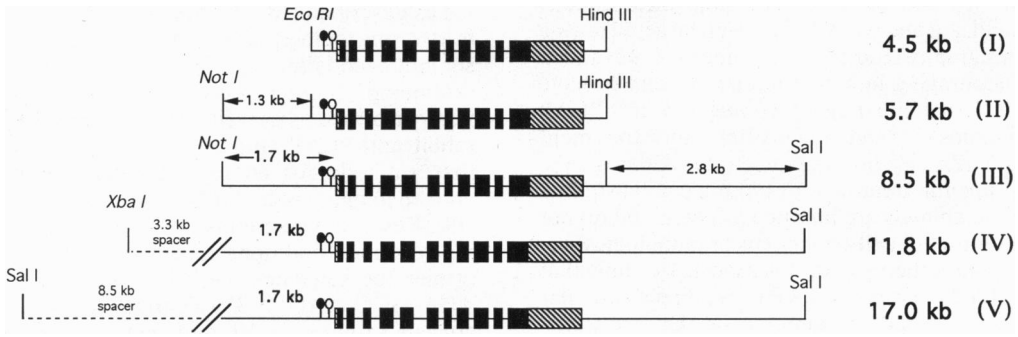

FIG. 1. Schematic representation of five GCAP DNA constructions used for microinjection to generate transgenic mouse lines. Solid boxes, protein coding exons; hatched boxes, 5′ and 3′ untranslated regions of exons (4). TATA box (open ellipsoid landmark) and Sp1 site (solid ellipsoid landmark) are indicated in the proximal 5′ flanking region of the constructs. Restriction sites were used to excise the DNA fragments from the cloning vectors. Size of constructions is indicated in kb and designation of constructions (I, II, III, IV, and V) is indicated in parentheses next to the size.

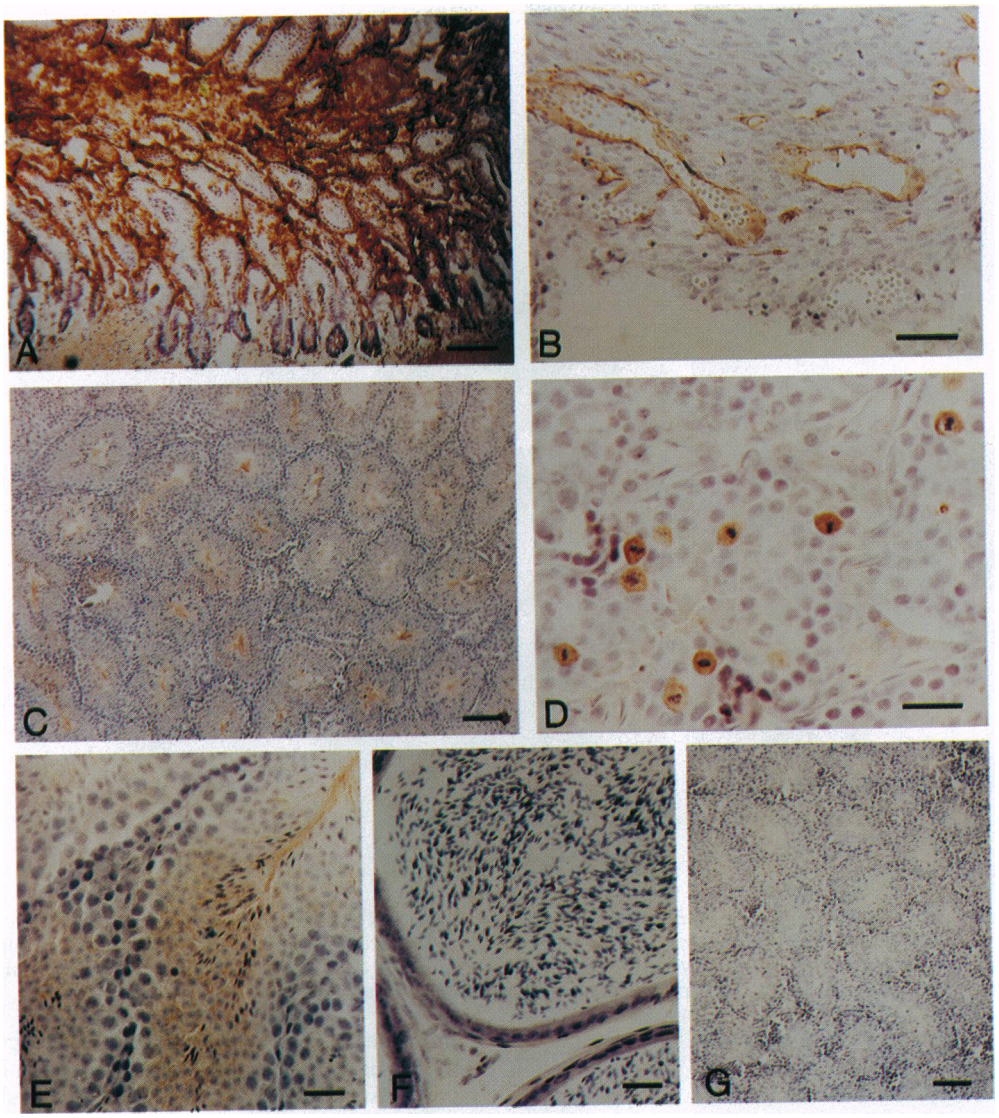

FIG. 2. Immunohistochemical staining for the presence of GCAP in transgenic mouse tissues. (*A*) Positively stained intestinal mucosa in a 7-d homozygote construction I mouse. (Bar = 100 μm.) (*B*) Positive endothelial cells in heart tissue from a 15.5-d construction I embryo. (Bar = 50 μm.) (*C*) Positive staining of seminiferous tubules in a 39-d construction V (P94 family) mouse. (Bar = 100 μm.) (*D*) Testis section of a 27-d construction V (P2 family) mouse showing positively stained spermatocytes in meiotic prophase I. (Bar = 25 μm.) (*E*) Testis of a 39-d construction V (P94 family) mouse showing positive GCAP staining in the membrane of spermatogenic cells and in the tails of spermatids. (Bar = 25 μm.) (*F*) Section through corpus section of epididymis of a construction V mouse (P2 family) showing negative GCAP staining of maturing sperm found in this region. (Bar = 25 μm.) (*G*) Testis section from a nontransgenic sibling (P2 family) showing no positive GCAP staining. (Bar = 100 μm.) Counterstaining was done with Mayer's hematoxylin.

lected to produce homogeneous cell populations with comparable isozyme contents (Fig. 4). FACS analysis of the relative binding of PLAP-positive and GCAP-positive MO4 cells with the conformational-specific anti-PLAP monoclonal antibodies 17E3, C2, and 7E8 indicated that the relative differences in affinity, and hence in conformation, were preserved for the cellular membrane-bound PLAP and GCAP (data not shown).

Whereas s.c. injection of untransfected MO4 cells gave rise to formation of tumors in syngeneic C3H mice and in control (C3H×FVB/N) hybrid mice (Fig. 5*A*), no tumor development could be observed when the cloned GCAP—or sorted PLAP—transfectants were injected in such animals. All attempts to experimentally induce immune tolerance for the

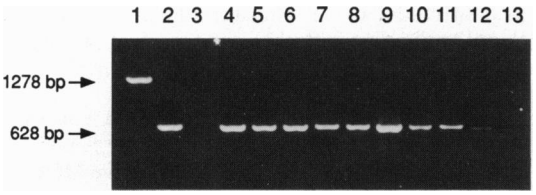

FIG. 3. RT-PCR amplification of GCAP mRNA. Lanes: 1, positive control for PCR using the 4.5-kb GCAP genomic clone in the vector KS+ Bluescript; 2, positive control for RT-PCR using RNA from the human GCAP-producing testicular cell line GERMA-1 (22); 3, negative control for RT-PCR using poly(A)$^+$ RNA from nontransgenic mouse testis; 4–10, RT-PCR product using poly(A)$^+$ RNA from transgenic mouse testis; 4, construction I (positive signal due to endothelial cell expression and not to germ cell-specific expression of GCAP); 5, construction II; 6, construction III; 7, construction IV; 8, construction V (P2 family); 9, construction V (P29 family); 10, construction V (P94 family); 11, RNA from eight-cell-stage construction V (P94 family) homozygous embryo; 12, RNA from construction V (P94 family) homozygous blastocysts; 13, RNA from construction V (P94 family) homozygous ovaries.

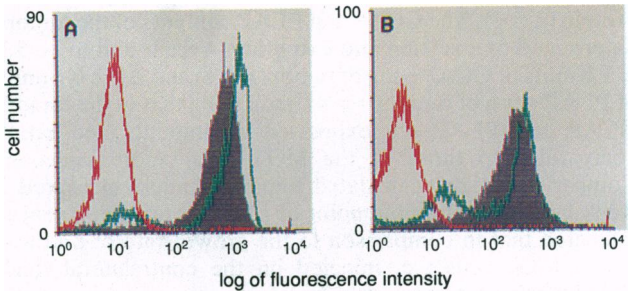

FIG. 4. FACS analysis of MO4 cells transfected with the gene encoding GCAP (*A*) or with the cDNA encoding PLAP (*B*). Profile of sorted cells upon control incubations with the secondary anti-mouse immunoglobulin antiserum exclusively (left red peak) and upon double labeling with the anti-GCAP/PLAP monoclonal antibody 7E8 and the secondary antiserum before (green profile) and after (solid gray area) subcloning of GCAP-positive cells and cell sorting of PLAP-positive cells.

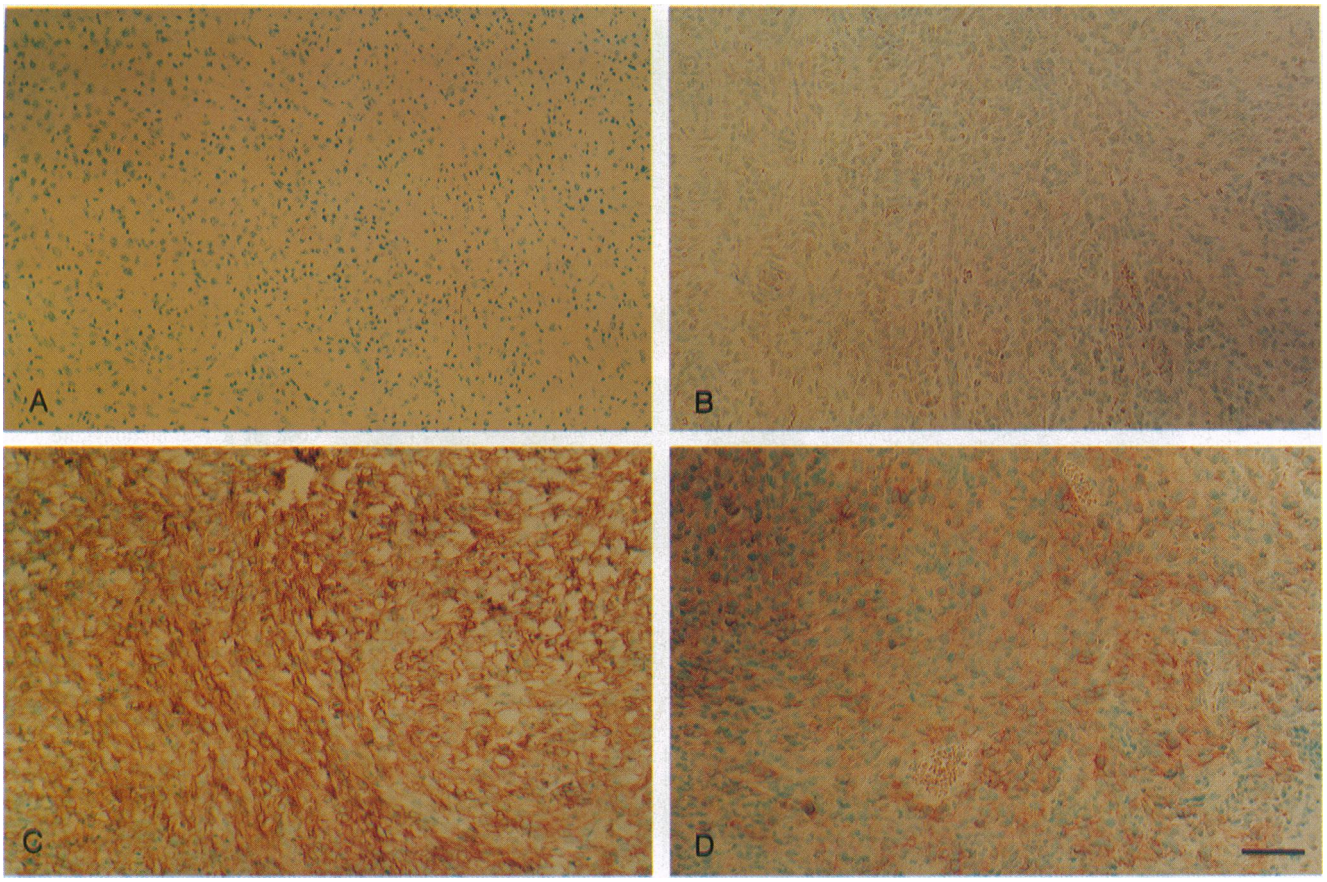

FIG. 5. Immunohistochemical staining with a polyclonal antiserum to GCAP and PLAP in sections derived from tumors grown in control (*A* and *B*) and GCAP transgenic (C3H×FVB/N) hybrid (*C* and *D*) mice. Tumors were obtained after s.c. injection of untransfected MO4 cells (*A*), of the mixture of transfected GCAP-negative and GCAP-positive MO4 cells before subcloning (*B*), of GCAP-positive MO4 cells upon subcloning (*C*), and of PLAP-positive MO4 cells upon FACS (*D*). Counterstaining was done with methyl green. (Bar = 100 μm.)

transfected cells in adult or neonatal mice were unsuccessful—i.e., they could not prevent T-cell-mediated tumor cell rejection. Upon injection of noncloned mixtures of transfected GCAP-negative and GCAP-positive cells, only GCAP-negative tumors developed (Fig. 5*B*). On the contrary, s.c. injection of cloned GCAP-positive MO4 cells in GCAP transgenic (C3H×FVB/N) hybrid mice invariably resulted in formation of a GCAP-positive tumor (Fig. 5*C*). Likewise, PLAP-positive tumors developed after s.c. injection in GCAP transgenic animals of sorted PLAP-positive MO4 cells (Fig. 5*D*). The specific enzyme activity of MO4 cells before injection was 0.48 ± 0.03 unit of GCAP per mg of cellular protein ($n = 8$) and 0.2 ± 0.02 unit of PLAP per mg of cellular protein ($n = 8$). The GCAP and PLAP contents of the tumors determined on excision and extraction were found to be 5.0 ± 1.8 units of GCAP per g of tumor ($n = 8$) and 2.0 ± 0.5 units of PLAP per g of tumor ($n = 4$), indicating that in the tumors GCAP and PLAP are expressed in amounts and ratios comparable to those of the MO4 cells before injection. Comparison of the calculated tumor volumes, measured 1 week later than the time point of palpable tumor presence, indicated that in comparison to the growth rate of untransfected MO4 cells s.c. injected on the contralateral thigh GCAP-positive tumors developed at a comparable rate in both male and female animals, homozygous or heterozygous (Table 1).

DISCUSSION

We aimed at generating transgenic mice that would express human GCAP tissue specifically in an attempt to establish an immunocompetent mouse model that would allow development of GCAP-producing syngeneic tumors. Two of our DNA constructions (constructions I and V) were successful in transcribing and expressing GCAP in the tissues of transgenic mice. Construction I allowed expression of GCAP in the intestinal epithelium and in endothelial cells. The high-level expression in these mice also resulted in high levels of circulating antigen. These transgenic mice, however, were not used further as an *in vivo* tumor model, since we reasoned that widespread expression of GCAP in the vascular epithelium and high antigenemia would represent adverse features of an animal model to be used for injection of anti-GCAP monoclonal antibodies with the purpose of immunolocalizing tumors. Possible deleterious side effects on the vascular

Table 1. Estimated tumor volumes for males and females, 1 week consecutive with the appearance of palpable tumors, following s.c. injection of subcloned GCAP-positive MO4 tumor cells in control, heterozygous, and homozygous GCAP (P2 family) transgenic (C3H×FVB/N)F_2 hybrid mice

	Calculated tumor volume, ml			
	Males		Females	
GCAP genotype	Control tumor	GCAP$^+$ tumor	Control tumor	GCAP$^+$ tumor
Negative	1.5 ± 0.4	—	2.9 ± 1.1	—
Heterozygous	2.4 ± 1.1	2.8 ± 1.1	2.3 ± 0.7	1.5 ± 0.7
Homozygous	2.0 ± 0.7	2.4 ± 1.4	3.0 ± 0.4	2.1 ± 0.8

Each group comprises a minimum of three animals. Values are means ± SD. Simultaneous injections of untransfected MO4 cells in contralateral thighs served as controls.

system and rapid elimination of antibody–GCAP complexes from the circulation would render such a model inadequate. On the other hand, construction V transgenic mice evidenced a very optimal pattern of expression—i.e., tissue expression confined to the testis in the adult animals, undetectable serum levels of the antigen, and embryonic GCAP expression that helped to establish immune tolerance for GCAP. An additional advantage of these transgenic mice is that the testis-specific GCAP expression mimics the normal site of GCAP expression in humans and, therefore, the potential teratogenic consequences of an immunotherapeutic approach to treating cancer could also be evaluated.

We stably transfected syngeneic MO4 fibrosarcoma cells with the cloned GCAP gene to be used for production of GCAP-positive tumors. Transfected murine MO4 cells expressing GCAP are rejected by syngeneic C3H mice. However, the immune rejection of GCAP-positive MO4 cells did not impair development of a tumor of untransfected MO4 cells. In (C3H×FVB/N) hybrid mice, expressing major histocompatibility complex antigens proper to the C3H mouse and transgenic for GCAP, GCAP-positive tumors can develop at a rate comparable to that observed for the untransfected control tumors. Hence, it is evident that the expressed transgene has granted cellular immune tolerance for GCAP to the experimental hybrid mice. This immune tolerance is already being ensured in the heterozygous state, which implies that further immunotherapeutical studies involving GCAP-positive MO4 tumors can be restricted to the offspring of normal C3H mice and homozygous GCAP FVB/N mice.

The expression of GCAP appeared to be confined to the testis—i.e., in the adult female no tissue could clearly be identified expressing the transgene. Yet, tolerance is equally evident in male and female animals, strongly suggesting that the immune tolerance for GCAP had already been established during embryological development (25). In agreement with the established immune tolerance in the GCAP transgenic animals, a morphological and immunohistochemical analysis of GCAP-positive tumor sections did not yield any evidence of T-cell infiltration. Macrophages and T cells were only found at the periphery of the tumor section (data not shown). We have shown that MO4 cells transfected with PLAP grow equally as well as GCAP transfected cells in GCAP transgenic mice. Yet, although PLAP and GCAP display a sequence identity of 98%, 10 amino acids are different between both mature isozymes (1, 2). These amino acid differences are responsible for a conformational difference between both molecules (20). Our present FACS analysis has confirmed that when anchored in the MO4 cellular membrane these structural differences are maintained. Inasmuch as single point mutations can lead to tumor rejection, it is remarkable that a difference of 10 amino acids in the PLAP-positive tumor cells does not lead to rejection by the GCAP transgenic animals. These findings substantiate that not all amino acid substitutions in a cancer cell's membrane are being recognized by the immune system.

This GCAP transgenic mouse model will allow evaluation of the immune responses accompanying repeated administration of conjugated or derivatized anti-GCAP and anti-PLAP monoclonal antibodies and can also be used to validate bifunctional antibodies with dual specificity for GCAP (and PLAP) and for the murine CD3 receptor to study *in vivo* the processes of antibody-mediated T-cell recruitment, targeting to MO4 tumors, metastases, and tumor cell lysis.

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