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Embryonal carcinoma antigen and the *T/t* locus of the mouse

(developmental genetics/immunology/preimplantation embryo)

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ABSTRACT **The presence of the F9 antigen and of four other antigens related to the *T/t* locus of the mouse was investigated by immunofluorescence on preimplantation embryos. In morulae heterozygous for any of these *t* haplotypes, both the appropriate *t* antigen and the F9 antigen are expressed. The F9 antigen segregates among the progeny of crosses producing embryos homozygous for some (t^{w32} and t^{w5}) but not for other haplotypes. It is concluded that (*i*) whatever the time of action of a *t* haplotype, its corresponding antigen is expressed during cleavage and (*ii*) the F9 antigen is specified by a gene(s) in the region of the *T/t* locus.**

Immunological study of embryonal carcinoma (EC) cells derived from a transplantable mouse teratoma allowed the detection of a new surface antigen(s), called "the F9 antigen" (1). This antigen, present on embryonal carcinoma cells, can be found on the cell surface of mouse preimplantation embryos. It is not detectable, however, on the somatic cells of the adult mouse—including thymocytes, brain and kidney cells—with the exception of spermatozoa and the whole male germ line (refs 1, 2, and unpublished data).

A segment of chromosome 17 of the mouse, known as the *T/t* locus, controls steps of embryonic development: a series of haplotypes derived from wild mice behave as recessive lethals, each one displaying a block of embryonic development at a particular stage (3). Each of these haplotypes is also known to specify some particular antigen(s) on the surface of sperm cells (3, 4). Thus, the genes at the *T/t* locus may act in embryonic development by controlling the synthesis of specific surface antigens required for cellular interactions at precise stages (5).

It was shown previously that the F9 antigen bears some relation with the *T/t* locus. The t^{w32} haplotype which in the homozygous condition prevents the transition from morula to blastocyst also affects the level of F9 antigen on sperm. To remove the same amount of anti-F9 antibodies, twice as many sperm cells are required from an heterozygous $+/t^{w32}$ as from a wild +/+ mouse (6). this result is consistent with the F9 antigen being determined by the wild counterpart of the t^{w32} haplotype; it is however compatible with other interpretations. We have, therefore, examined this problem further by investigating the presence of the F9 antigen and of certain *t* antigens on preimplantation embryos produced in crosses involving *t* haplotypes. If the F9 and the *t* antigens are products of the *T/t* complex, they should be expressed codominantly and should segregate among the progeny of appropriate crosses.

MATERIALS AND METHODS

Mice. The following stocks were used: 129/Sv, an inbred subline of the original 129 strain; NCS, a random bred strain of albino mice; BT BRTF/Nev, a moderately inbred line on which *t* haplotypes are maintained in balanced lethal crosses $T/t^x \times T/t^x$. All mice were produced at the Institut Pasteur, except for the BT BRTF/Nev T/t^{w5} females which were obtained from Drs. K. Artzt and D. Bennett, Cornell University, N.Y., N.Y. All stocks that carried *t* haplotypes were received in 1972 from Dr. D. Bennett.

Heterozygous embryos $+/t^x$ or $T/+$ were produced by backcrossing F_1 hybrids to wild-type parents—e.g., $+/t^x$ embryos issued from a cross: ♀ 129/Sv +/+ × ♂ F_1 (129/Sv × BT BRTF) $+/t^x$. Homozygous t^x/t^x embryos were produced by either backcrossing the F_1 hybrids to the BT BRTF T/t^x stock or by intercrossing $+/t^x$ mice in which a particular t^x haplotype had been backcrossed five to eight times on the 129 background.

Cells. Two cultured cell lines were used: F9-41, a nullipotential embryonal carcinoma cell (1); PYS-2, a parietal yolk sac cell (7) devoid of F9 antigen.

Antisera. Anti-F9 Serum. Anti-F9 sera were produced in syngeneic male 129/Sv mice by hyperimmunization with irradiated F9-41 cells (1). Before its use on embryos, anti-F9 serum was absorbed with PYS-2 cells (vol/vol, serum diluted 1 to 5 in Hanks' medium containing 4% heat-inactivated fetal calf serum, γ-globulin free, for 1 hr at 0°), and then on lymph nodes lymphocytes of the same genotype as the embryos to be studied (vol/vol, serum diluted 1 to 15, for 1 hr at 0°). The absorbed sera retained most of the original activity on F9-41 cells: in microcytotoxicity tests, 90–95% of the cells were killed with a titer of 1:2400 to 1:3200; in indirect immunofluorescence, 100% of F9-41 cells were labeled up to a 1:400 dilution.

Anti-t^{w32} Serum. C57BL/6 males were immunized with spermatozoa of T/t^{w32} animals (4). After six to seven immunizations, the sera were collected and heat inactivated. They were sequentially absorbed with +/+ (129 and C57BL/6) and $T/+$ (BT BRTF/Nev) testicular cells and spermatozoa (vol/vol, serum diluted 1 to 5, for 1 hr at 0°). The absorbed anti-t^{w32} serum was then diluted 1:10 and further absorbed with lymphocytes from T/t^{w32} mice. The anti-t^{w32} serum was tested on spermatozoa by indirect immunofluorescence: 35–40% spermatozoa of $+/t^{w32}$ animals were stained up to a 1:60 dilution. Only the postacrosomal zone was stained, a location identical to that found with anti-F9 serum (8). At a 1:5 dilution, the anti-t^{w32} serum failed to stain spermatozoa of +/+, $T/+$, T/t^o and T/t^{w5} animals. It was no longer toxic towards F9 cells and BT BRTF lymphocytes. No specific labeling was detected on F9 cells at a 1:10 dilution.

Antisera against T/t^{w5}, T/t^o, and $+/t^{w18}$ Sperms. These antisera were a gift of Dr. K. Artzt. The three sera were absorbed with sperm from +/+ (129) and $T/+$ (F_1 129 × BR BRTF) mice and with lymphocytes from mice carrying the appropriate *t* haplotype as described for anti-t^{w32} sera.

Anti-Mouse Ig Serum. Rabbit anti-mouse Ig serum labeled with fluorescein was a product of Institut Pasteur Production, Paris. Before use, it was massively and repeatedly absorbed with F9-41 and PYS-2 cells. The absorbed conjugates were sampled and stored frozen at −28°. They were used at a 1:25 dilution in Whitten's medium (9).

Immunolabeling of Preimplantation Embryos. Mouse embryos were flushed with Whitten's medium from the uterus

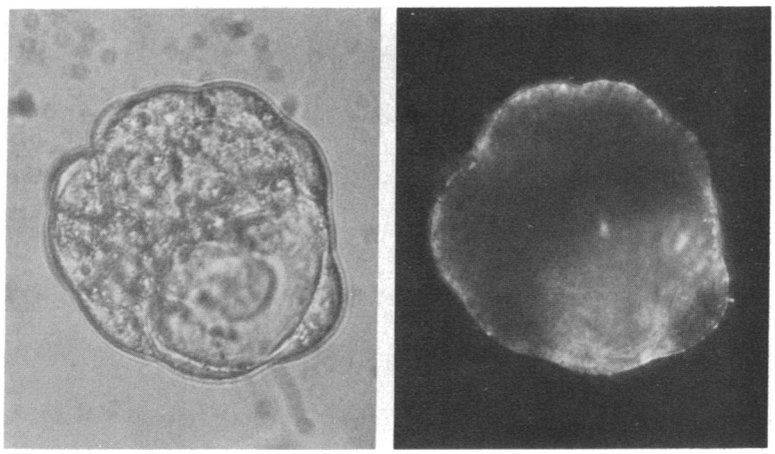

FIG. 1. Immunolabeling of a 129/Sv blastocyst with anti-F9 serum, $1/160$ dilution. Left, phase contrast; right, fluorescence. Magnification, ×1000.

during day 3 (morulae) or day 4 (blastocysts), detection of vaginal plugs being taken as day 1. Spontaneously ovulated and hormone-primed females (five international units of pregnant mare serum followed 40 hr later with five international units of human chorionic gonadotropin) were used with the same results. Immediately after the embryos were flushed, the zona pellucida was removed with Pronase (10); the embryos were incubated for 2 hr in Whitten's medium under a 5% CO_2-95% air atmosphere at 37°.

Immunolabeling was carried out in microplates (Falcon) for 1 hr at 4° in a reaction volume of 15 μl. The embryos were washed three times in Whitten's medium, incubated with the fluorescein conjugated anti-mouse Ig (1 hr, 4°), washed three times in Whitten's medium, and placed in phosphate-buffered saline for examination under a fluorescence microscope. Photographs were taken with an Ilford HP4 film developed at 800 ASA.

RESULTS

Coexpression of F9 antigen and of several *t* antigens on appropriate heterozygous morulae

Indirect immunofluorescence allows an easy detection of the F9 antigen on early embryos a few hours after fertilization. With up to a 1:320 dilution of anti-F9 serum, 95% or more wild-type morulae or blastocysts were labeled. Both nonimmune serum and anti-F9 serum absorbed with F9 cells give no staining even at a 1:10 dilution. This applies to several strains tested whether inbred (129/Sv, C57BL/6, SJL/J) or outbred (NCS). Typical staining of a blastocyst is shown in Fig. 1. In all experiments, the anti-F9 serum was used at a 1:100 dilution.

The various *t* antigens are defined on sperm (4); they have not yet been investigated on embryos. We have looked for the presence of several t^x antigens and of F9 antigen on heterozygous $+/t^x$ morulae. The presence of the t^{w32} antigen, for instance, was investigated on 8-cell stage morulae from crosses between +/+ 129/Sv females and $+/t^{w32}$ males (on 129/Sv background). As shown in Table 1, the anti-t^{w32} serum labeled 65 out of 118 embryos tested (55%), the embryos being unambiguously positive or negative (Fig. 2). Controls with anti-t^{w32} serum absorbed with sperm from $+/t^{w32}$ mice did not label any embryo (data not shown), nor did anti-t^{w32} serum label any +/+ embryo in control crosses.

In the same way, anti-t^{w5}, anti-t^o, and anti-t^{w18} sera were used to label heterozygous embryos obtained by crossing +/+ 129/Sv females with $+/t^{w5}$, $+/t^o$ or $+/t^{w18}$ males respectively (Table 1). In every case, a sizable fraction of the embryos (53, 25 and 46%) was labeled by the corresponding antiserum. The progenies of similar crosses were also tested for the presence of F9 antigen. In all cases, nearly all the embryos tested were labeled with anti-F9 serum (Table 1).

From these experiments, it can be concluded that: (*i*) among the *t* antigens previously detected on sperm, the four tested are expressed on appropriate heterozygous embryos; (*ii*) though the different *t* haplotypes block development at various stages, these four antigens are already expressed at the eight-cell stage; (*iii*) in heterozygous morulae, each of these *t* antigens is co-expressed with F9 antigen; and (*iv*) the presence of these *t* antigens on eight-cell morulae results, not from unmasking of some previous structure, but from post-zygotic synthesis since the *t* haplotype always comes from the male.

Segregation of the F9 antigen in the progeny of crosses between two mice heterozygous for a same *t* haplotype

Because F9 and every *t* antigen tested are co-expressed on suitable heterozygous embryos, it is possible to follow their distribution among the progeny of crosses between two mice which are heterozygous for a same *t* haplotype. Crosses $+/t^x \times +/t^x$ produce embryos of the three classes: +/+, $+/t^x$, t^x/t^x. The t^x antigen should be absent from +/+ embryos and,

Table 1. Immunolabeling of morulae produced in crosses ♀129/Sv +/+ × ♂$+/t^x$

Anti-serum (dilution)	Genotype of the males				
	+/+	$+/t^{w32}$	$+/t^{w5}$	$+/t^0$	$+/t^{w18}$
Anti-F9 (1/100)	88/92*	41/42	22/22	19/19	66/68
Anti-t^{w32} (1/12)	0/54	65/118	—	—	—
Anti-t^{w5} (1/8)	0/18	—	18/34	—	—
Anti-t^0 (1/18)	0/20	—	—	17/68	—
Anti-t^{w18} (1/12)	3/21	—	—	—	42/91

Males were either F_1(129 × BT BRTF/Nev) hybrids, or products of successive backcrosses (5–8) on 129 background. Similar results were obtained in both cases.

* Number of labeled embryos/number of total embryos examined.

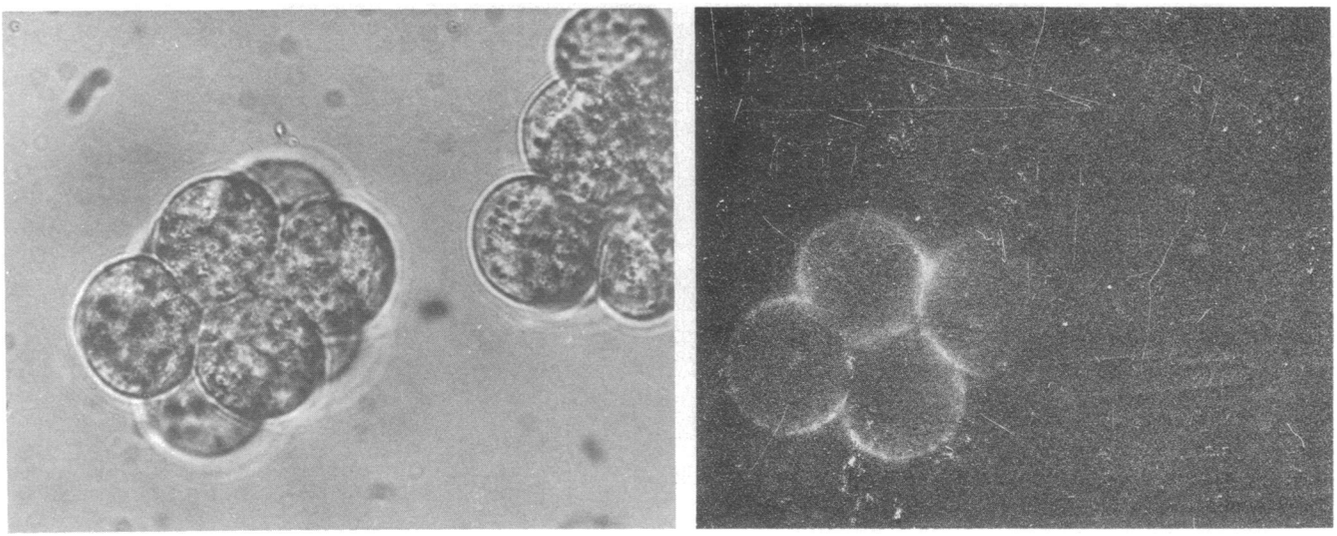

FIG. 2. Immunolabeling of morulae from a cross ♀ 129 +/+ × ♂ $+/t^{w32}$ F_1(BT BRTF.129) with anti-t^{w32} serum, 1/12 dilution. Left, phase contrast; right, fluorescence. Note the t^{w32}-negative morula (presumably +/+) in the upper right corner. Magnification, ×1000.

therefore, segregate among the progeny. If the F9 antigen is determined by a gene unrelated to the T/t complex, then it should either not segregate among the progeny or segregate randomly with respect to the t^x antigen. If, however, the F9 antigen is determined by the wild T/t haplotype, it might be either altered or lacking in one or several t^x haplotypes and then be absent from t^x/t^x embryos. In crosses involving such t^x haplotypes, there should be a class of embryos unlabeled with anti-t^x serum and a distinct class unlabeled with anti-F9 serum.

t^{w32} Haplotype. The expression of F9 and t^{w32} antigens was investigated in the progeny of different crosses (Table 2).

(*i*) $+/t^{w32}$ females F_1(+/+ ♀ NCS × T/t^{w32} ♂ BR BRTF/Nev) × T/t^{w32} males BT BRTF/Nev. The embryos were examined during day 3 or 4. During day 3, most of the embryos, including t^{w32}/t^{w32} homozygotes, reached the early morula stage. Only morulae were analyzed, and uncleaved eggs were discarded. With anti-F9 serum, only 17 out of 26 embryos (65%) were labeled. In separate experiments with anti-t^{w32} serum, 19 out of 32 embryos (61%) were labeled.

During day 4, most of the +/+ and $+/t^{w32}$ embryos should reach the blastocyst stage, while all the t^{w32}/t^{w32} embryos are blocked at the morula stage (3). Thus, blastocysts are either +/+ or $+/t^{w32}$, whereas the majority of morulae should be t^{w32}/t^{w32}. The progenies were indeed found to be composed of 65% blastocysts and 35% morulae. Out of 127 embryos tested, 45 were unlabeled with anti-F9 serum, and were all morulae. In two experiments with anti-t^{w32} serum, 11 of 18 embryos were unlabeled, and were mostly blastocysts (this abnormally high ratio was due to the progeny of one mouse described in Table 3).

(*ii*) Females BT BRTF/Nev T/t^{w32} × males BT BRTF/Nev T/t^{w32}. Similar experiments with anti-F9 serum were carried out on preimplantation embryos from balanced lethal crosses and examined during day 4 with similar results (Table 2).

These experiments with individual sera do not show whether the embryos unlabeled with one serum can be labeled with the other. Because of technical difficulties, double immunolabeling could not be performed. Instead, the following procedure was used. In a first step, the embryos (day 4) were exposed to one serum and immunologically stained. Unlabeled embryos were then removed and treated with the other serum. As shown in Table 3, lines 1 and 2, those embryos not labeled with anti-F9 serum—again all morulae—were all labeled with anti-t^{w32} serum and vice versa. Under the conditions used, the two classes of unlabeled embryos do not overlap.

There is no way of correlating conclusively the genotypes and the phenotypes of the embryos. These results, however, conform entirely to the expectation that t^{w32}-negative embryos are +/+ homozygotes—because they belong mainly to the blastocyst class—and that F9-negative embryos are t^{w32}/t^{w32} homozygotes—because they belong exclusively to the morula class. In

Table 2. Labeling of embryos from crosses producing t^{w32}/t^{w32} homozygotes by anti-F9 and anti-t^{w32} sera

Antiserum (dilution)	Experimental crosses: ♀ $+/t^{w32}$ × ♂ BT BRTF T/t^{w32}		Experimental crosses: ♀ BT BRTF T/t^{w32} × ♂ BT BRTF T/t^{w32}	Control crosses: ♀ 129 +/+ × ♂129 +/+
	Day 3	Day 4	Day 4	Day 3 or 4
Anti-F9 (1/100)	9/26*	45/127	14/31	4/92
Anti-t^{w32} (1/12)	12/31	11/18	N.T.	38/38

Embryos were obtained at day 3 (morulae) or day 4 (late morulae or blastocysts). At day 4, the anti-F9 serum labeled all the embryos which had developed into blastocysts, and also some which had developed into late morulae. F9-negative embryos at day 4 were always found to be morulae. N.T., not tested.

* Number of unlabeled embryos/total number of embryos examined.

Table 3. Sequential labeling of embryos from crosses producing t^{w32}/t^{w32} or t^{w5}/t^{w5} homozygotes

Crosses* ♀	Crosses* ♂	Number of embryos	First labeling with antiserum	Results of first labeling +	Results of first labeling —	Second labeling of negative embryos with antiserum	Results of second labeling +	Results of second labeling —
$+/t^{w32}$	T/t^{w32}	17	Anti-F9	8	9	Anti-t^{w32}	9	0
$+/t^{w32}$	T/t^{w32}	11	Anti-t^{w32}	2	9	Anti-F9	9	0
T/t^{w5}	$+/t^{w5}$	41	Anti-F9	23	18	Anti-t^{w5}	18	0

* Same crosses as in Tables 2 and 4.

that case it can be concluded that (*i*) the t^{w32} haplotype does not produce the F9 antigen and (*ii*) the F9 antigen segregates as expected for a product of the "wild *t* haplotype."

t^{w5}, t^{w18}, and *T* Haplotypes. For any t^x haplotype other than t^{w32}, there is no way of distinguishing morphologically t^x/t^x preimplantation embryos since the block in development always occurs after implantation (3). Anti-F9 serum was used to label morulae produced in crosses between two mice both heterozygous for one of the haplotypes t^{w5}, t^{w18}, or *T*. The results are summarized in Table 4. In crosses producing t^{w18}/t^{w18} or T/T homozygotes, nearly all embryos were labeled, as they were in control crosses producing T/t^{w5} heterozygotes. In contrast, in crosses producing t^{w5}/t^{w5} homozygotes, 34% of the embryos were not labeled. In the latter case, sequential labeling (Table 3, line 3) showed that F9-negative embryos were all labeled by anti-t^{w5} serum. Thus, the F9 antigen appears to be produced by two haplotypes (t^{w18} and *T*) but not by two others (t^{w32} and t^{w5}).

DISCUSSION

A word should first be said about the immunological system used. Allogeneic anti-t^x sera are prepared by injecting sperm from T/t^x mice of various genetic background in C57BL/6 males. Absorption with sperm from +/+ and $T/+$ mice leaves an activity specific for the sperm of mice carrying a t^x haplotype (4). However, recessive lethal *t* haplotypes are obtained, not by mutation, but from natural populations (3); most of them do not produce recombinants over a large segment of chromosome 17 (3) and each is found associated with a particular H-2 haplotype (11). In our experiments, we have removed activities such as anti-H-2 by absorbing with lymphocytes from mice carrying the same t^x haplotype. Yet one cannot exclude that an anti-t^x serum might detect, in addition to a genuine t^x antigen, some other unknown antigen specified by a gene linked to the T/t locus.

Table 4. Labeling by anti-F9 serum of embryos from crosses producing t^x/t^x or T/T homozygotes, and T/t^{w5} heterozygotes

Antiserum (dilution)	Crosses producing embryos* t^{w32}/t^{w32}	t^{w5}/t^{w5}	t^{w18}/t^{w18}	T/T	T/t^{w5}
Anti-F9 (1/100)	68/188†	46/135	2/54	4/84	1/29

* t^{w32}/t^{w32}: summary of data from Table 2. t^{w5}/t^{w5}: cross ♀ BT BRTF/Nev T/t^{w5} × ♂ BT BRTF/Nev T/t^{w5} or ♀ BT BRTF/Nev T/t^{w5} × ♂ F_1(129.BT BRTF) $+/t^{w5}$. t^{w18}/t^{w18}: cross ♀ 129 N5 $+/t^{w18}$ × ♂ 129 N5 $+/t^{w18}$. T/T: cross ♀ 129 N8 $T/+$ × ♂ F_1(BT BRTF.129) $T/+$. T/t^{w5}: cross ♀ 129 N8 $T/+$ × ♂ F_1(BT BRTF.129) $+/t^{w5}$ (control).

† Number of unlabeled embryos/total number of embryos examined.

The experiments reported in this paper show that the *t* antigens defined on sperm are indeed expressed on embryos carrying the appropriate *t* haplotype. A sizable fraction of embryos from crosses which yield $+/t^x$ heterozygotes are specifically labeled by anti-t^x sera [the fraction of labeled embryos is not quite as large as one would expect from the high transmission ratios (3) of t^{w5} and t^{w32}; this quantitative discrepancy may arise from any of several causes].

The coexpression of F9 antigen and of *t* antigens represents the net contribution of both parents. This indicates that these antigens do not result from unmasking or modification of preexisting structures but involve gene products synthesized after fertilization. However, the *t* antigens are not expressed in sequence during development as generally assumed from the sequence of lethal effects caused by the various *t* haplotypes in homozygous embryos. Instead, the four tested (and perhaps all) *t* antigens are expressed during cleavage. This does not exclude their role in cellular interactions during specific stages of development but more complex models are then required.

Because surface antigens determined by *t* haplotypes are expressed on embryos, it is reasonable to assume that similar antigens are specified by the "wild haplotype." This is what the F9 antigen appears to be. Its distribution among the progeny of crosses producing homozygous embryos indicates that it is specified by a region belonging, or closely linked, to the T/t locus. One might argue that the segregation of F9-negative embryos is due to some heterogeneity in the animal stocks or to some damage caused to the membrane by certain *t* haplotypes. The fact that in a cross producing t^{w32} or t^{w5} homozygotes every F9-negative embryo has so far been stainable by the appropriate anti-*t* (t^{w32} or t^{w5}) serum, and conversely every t^{w32}-negative embryo by anti-F9 serum, is a strong indication that the observed segregation does express the segregation of the *t* haplotypes. The F9 antigen appears to be altered or absent in some *t* haplotypes (t^{w32} or t^{w5}) but not in two others (t^{w18} or *T*). The former result prevents, for the time being, the F9 genetic determinant from being considered as the "wild allele" of a particular *t* gene and the F9 antigen from being assigned any particular function.

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