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Presence of the Adenovirus E1A-Like Activity in Preimplantation Stage Mouse Embryos

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The presence of the adenovirus E1A-like activity in embryonal carcinoma stem cells has been reported. We now show that preimplantation stage mouse embryonic cells allow transcription of the E1A-dependent E2A gene when infected with E1A-deleted mutant *dl*312, indicating the presence of the E1A-like activity in morulae and blastocysts. Moreover, such activity seems to decrease or disappear at about the time of implantation.

Adenovirus E1A gene products immortalize primary rodent cells and work as the *trans*-acting regulators which activate or repress transcription of other adenovirus genes and many cellular genes (12), such as heat shock proteins (7) and major histocompatibility complex antigen genes (14, 19). Presence of the E1A-like activity in embryonal carcinoma (EC) stem cells was shown by infection of the stem cells with E1A-deleted an adenovirus type 5 (Ad5) mutant (7), *dl*312, and by studies on regulation of virus gene expression, such as by polyomavirus and its mutants (5). The presence of the E1A-like activity in undifferentiated EC stem cells and its disappearance after induced differentiation imply an important role for the E1A-like activity in cell differentiation (8, 10, 13). However, it is not known whether the activity is related more to the undifferentiated state or to the transformed phenotype of the EC cells. Such implications would become much more important if the E1A-like activity were present not only in the tumor cells but also in the undifferentiated cells of normal early embryos.

The presence of an E1A-like activity in EC cells has been shown by the following two lines of evidence. The E1A-dependent E2A gene (9) is transcribed when EC stem cells are infected with *dl*312 (7). However, the E2A gene is not activated when infected into differentiated derivatives of EC stem cells (7). More recently, the presence of cellular factors responsible for the stimulation of E2A transcription was demonstrated in EC stem cells but not in differentiated cells (10, 13). Another line of evidence is that several viral genomes transcribed by the use of enhancers from polyomavirus, simian virus 40, and Moloney murine leukemia virus, all of which are repressed by the E1A gene products (2, 5), are repressed in EC stem cells but efficiently transcribed in differentiation-induced EC cells (3, 6, 11, 16). Moreover, mutations in the polyomavirus enhancer which allow expression of the virus genomes in EC stem cells are also tolerant to repression of E1A gene products (5). We used infection of mouse early embryonic cells by the mutant *dl*312 to study the presence of the E1A-like activity.

ICR strain female mice were superovulated by injecting 5 U of human chorionic gonadotropin (hCG) per mouse at 48 h after injection of 5 U of pregnant-mare serum gonadotropin (PMSG). Immediately after the injection of hCG, females were caged with ICR male mice. Females with a vaginal plug were designated to be 0.5-day pregnant at noon. Eight-cell-stage embryos were collected by flushing oviducts with PB1 solution (21) at 2.5 days. Blastocysts were flushed from uteri at 3.5 days. After removing zona pellucidae by treatment with acidic Tyrode solution, embryos were infected with Ad5 or *dl*312 in M16 medium (20) at a concentration of 5×10^7 PFU/ml. Denuded embryos were incubated for 1 h in 40-μl drops (5 embryos per drop) of the virus suspension on a petri dish bottom covered with liquid paraffin oil before the addition of 4 μl of fetal bovine serum to each drop. In the positive-control experiments, we observed efficient infection of embryos and cultured cells with the wild-type virus, judging from the production of E2A protein. For example, 80 to 100% of the 3.5-, 4.5-, and 5.5- to 6.5-day-old embryos were successfully infected under these conditions (Table 1). The infected embryos were cultured for 2 days in a CO_2 incubator at 37°C. Embryos were fixed with 2% paraformaldehyde solution in phosphate-buffered saline (PBS) for 30 min at 4°C and permeabilized with 1% Nonidet P-40 solution in PBS for 20 min at room temperature (RT). They were transferred onto glass slides, air dried, treated with cold methanol for 10 min, and air dried again. They were rehydrated in PBS for 10 min and incubated with anti-E2A rabbit serum (17) for 1 h at RT. After being rinsed in PBS for 1 h with several changes of PBS, samples were incubated with the fluorescein isothiocyanate-conjugated secondary antibody for 1 h at RT.

Denuded 8-cell-stage embryos and morulae (2.5 days) were infected with *dl*312 (1, 15) under the conditions described above. The embryos showed no apparent damage, in contrast to those infected with the wild-type virus, which caused developmental arrest and embryo death (data not shown). The E2A product was not detectable at 1 day postinfection by immunofluorescence staining with an antiserum (17) against the E2A protein. However, at day 2, more than half of the embryos were positively stained (Fig. 1a and Table 1). Production of the 72-kilodalton (kDa) E2A DNA-binding protein was confirmed by Western blot (immunoblot) analysis with the same antiserum (Fig. 2).

As the next step, we tried to determine whether the E1A-like activity decreased or disappeared when embryonic development proceeded further. As shown in Fig. 1c and Table 1, 3.5-day blastocysts infected with *dl*312 showed positively stained nuclei. By use of immunosurgery, we isolated the inner cell mass and infected it with viruses, confirming positive staining of the inner cells as well as the outer trophectoderm cells. However, such staining decreased drastically when 4.5-day expanded or hatched blastocysts were infected (Fig. 1d and Table 1). Outgrowth

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TABLE 1. E2A expression in embryos infected with *dl*312 or wild-type Ad5[a]

Infected stage (days)	Virus	No. of embryos positive/no. tested	% Positive
2.5	*dl*312	5/8	63
	None	0/9	0
3.5	*dl*312	11/18	61
	Ad5	10/10	100
4.5	*dl*312	2/14	14
	Ad5	9/9	100
5.5–6.5	*dl*312	0/7	0
	Ad5	4/5	80
7.5–8.5	*dl*312	0/10[b]	0

[a] An embryo with at least one positively stained nucleus was counted as a positive embryo. Embryos were infected and prepared for immunohistochemistry with anti-E2A antiserum as described in the text.

[b] E2A gene expression was not detected by screening more than 10 colonies of the embryonic ectoderm cells.

from the attached blastocysts, which were cultured for 3 days from the 3.5-day blastocysts before infection and corresponded to the 5.5- to 6.5-day-implanting blastocysts, showed no staining, nor did the explanted embryonic ectoderm cells which were isolated from the 7.5-day primitive-streak-stage embryos by microsurgery, as described previously (4) (Table 1). However, embryos at these stages (3.5 to 6.5 days) produced the E2A protein after wild-type Ad5 infection (Fig. 2b and Table 1), ensuring that the lack of E2A expression in the *dl*312-infected embryos was not simply due to the lack of virus infection.

These results show that an adenovirus E1A-like activity is present in preimplantation stage mouse embryos. We do not know whether the E1A-like activity observed in embryos is the same as that observed in mouse EC stem cells. As a possible approach to determine their similarities, it would be of interest to test whether the early embryos or the cell extracts are able to suppress the viral enhancers. In any case, the present finding increases the importance of the E1A-like activity or factors found in EC stem cells, which have been used as a model system for differentiation from pluripotent undifferentiated stem cells in early embryos into more fate-limited differentiated cells.

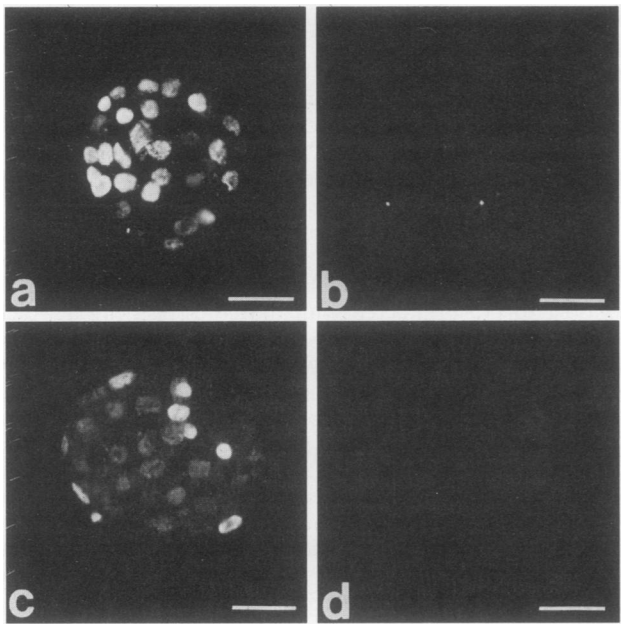

FIG. 1. Immunofluorescence histochemistry of embryos, stained with anti-E2A antiserum after being infected with *dl*312 and cultured for 2 days. (a) One of the embryos infected at the eight-cell or morulae stage (2.5 days). Note positively stained nuclei. (b) Mock-infected (2.5-day) control embryo. (c) Blastocyst (3.5 days) showing positive staining in nuclei. (d) An expanded or hatched blastocyst (4.5 days) with almost no staining. Bars, 50 μm.

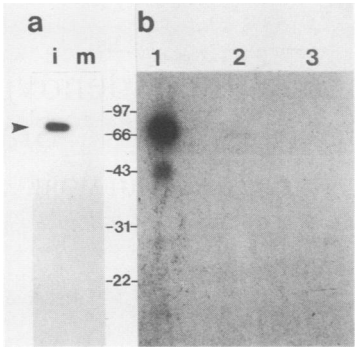

FIG. 2. (a) Western blot analysis of 2.5-day embryos infected with *dl*312 and stained with anti-E2A antiserum. A large number of 2.5-day embryos were infected with viruses as described in the text and collected in sample buffer after being cultured for 2 days. Cell extracts from 30 embryos were applied to each lane. The 72-kDa E2A protein band (arrowhead) was seen in infected embryos (lane i) but not in mock-infected embryos (lane m). Positions of molecular weight markers (in kilodaltons) are shown on the right. (b) Similar blot analysis of 5.5- to 6.5-day embryos infected with wild-type Ad5 (lane 1) and 4.5- (lane 2) or 5.5- to 6.5-day (lane 3) embryos infected with *dl*312. Infection by the wild-type virus resulted in a large amount of the E2A gene product, while infection by *dl*312 produced almost no E2A protein. Western blotting was carried out as described before (18). Samples were electrophoresed in a 12% polyacrylamide gel and electrophoretically blotted onto a nitrocellulose membrane. The membrane was treated with the primary antiserum, washed, treated with ^{125}I-labeled protein A, and processed for autoradiography.

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