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Evidence for Two Points of Restriction in the Expression of Adenovirus Type 2 in Cultured Epidermal Keratinocytes

BRIAN J. ANESKIEVICH AND LORNE B. TAICHMAN*

Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-8702

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Cultures of epidermal keratinocytes contain two populations of cells, a basal undifferentiated population and a suprabasal terminally differentiated population. When exposed to wild-type adenovirus type 2 (wtAd2), the suprabasal cells are positive by immunofluorescence for capsid antigen and exhibit cytopathic effects (CPE) (R. F. LaPorta, and L. B. Taichman, Virology 110:137–146, 1981). The basal cells, although infected, are not positive for capsid antigen and do not display CPE. Despite CPE and capsid antigens in suprabasal cells, yields of virus from the entire culture are very low (10 PFU per cell). These observations suggest that Ad2 expression is restricted at different times in the viral life cycle in basal and suprabasal cells. To test this hypothesis, we isolated host range (*hr*) mutants of Ad2 on two lines of squamous cell carcinoma (SCC) keratinocytes which were shown to be restrictive for wtAd2 replication. The hrAd2 mutants produced high yields of progeny virus in epidermal cell cultures (500 to 600 PFU per cell). However, the pattern of CPE induction in these cultures was like that produced by wtAd2, i.e., basal cells were CPE negative and suprabasal cells were CPE positive. The high yield of *hr*Ad2 progeny indicated that the restriction present in suprabasal cells was overcome. However, the failure of *hr*Ad2 mutants to induce CPE in basal cells indicated that the *hr*Ad2 mutants remain restricted in the basal population and supported our hypothesis that a second and distinct restriction exists in basal keratinocytes.

Epidermal keratinocytes grow in culture to produce a stratified squamous epithelium consisting of two populations of cells, a replicating population located primarily in the basal layer and a terminally differentiating population located in the suprabasal layers (1, 11). When such cultures are infected with adenovirus type 2 (Ad2), there is a marked difference in the response of basal cells and suprabasal cells (14). Suprabasal cells exhibit typical cytopathic effects (CPE) and synthesize capsid proteins as detected by immunofluorescence, whereas basal cells appear normal, continue to replicate, and are not positive for capsid antigens. Direct measurement in differentiated and undifferentiated keratinocytes has confirmed a higher rate of capsid protein synthesis in the differentiated cell population (B. A. Aneskievich, J. I. Lee, and L. B. Taichman, unpublished data). Despite the capsid antigen and a well-developed CPE in suprabasal cells, the yield of progeny virus on a culture-wide basis is only about 10 PFU per cell (4a). These results indicate that Ad2 expression is restricted in suprabasal cells at a point in the viral life cycle after the onset of capsid protein synthesis but before production of high titers of progeny virions. The facts that basal cells fail to stain for viral capsid proteins and do not exhibit CPE suggest that Ad2 expression is also restricted in basal cells but at a different and earlier point in the virus life cycle.

To test whether Ad2 expression is restricted in different ways in basal and suprabasal cells, we posed the following question: Will host range (*hr*) mutants of Ad2 which produce high yields of infectious virus in cultures of epidermal keratinocytes overcome the restriction in both basal and suprabasal cells? If the restriction to wild-type (wt) Ad2 expression is the same in both basal and suprabasal cells, we expect that hrAd2 mutants will induce similar responses in the two cell types. If restriction is different in the two cell types, the responses to infection with *hr*Ad2 are likely to be different.

*hr*Ad2 mutants were selected by serial passage of wtAd2 in SCC12B2 and SCL1 cells. SCC12B2 and SCL1 are lines of human keratinocytes established from epidermal squamous cell carcinomas (SCC) (6, 19). Unlike normal keratinocytes which senesce, have a low plating efficiency (20), and are composed of a heterogeneous population of differentiating and nondifferentiating cells (1), SCC cells are immortalized, easily cloned (19), and homogeneous with respect to differentiation (18). These SCC lines also restricted growth of wtAd2 as evidenced by the lack of CPE (Fig. 1E) and the low yields of progeny virus (range, 29 to 126 PFU per cell; Table 1). A growth curve of wtAd2 in SCC12B2 and normal keratinocytes showed that kinetics of viral replication are very similar in the two cell types and confirmed that titers measured 3 days postinfection (p.i.) are, in fact, derived from progeny virus (Fig. 2).

An initial stock of plaque-purified wtAd2 was mutagenized with 0.7 M nitrous acid in 1.0 M acetate buffer (9) with subsequent modifications (12, 24). SCC12B2 and SCL1 cells cultured as described elsewhere (19) were infected with mutagenized and nonmutagenized wtAd2 (0.05 PFU per cell). A lysate of the culture was prepared 3 days p.i. by freeze-thawing, and a titered sample was used to initiate another cycle of infection. After three or four passages, CPE was noted in the SCC12B2 and SCL1 cultures infected with mutagenized and nonmutagenized virus. Mutants were purified from each isolate by two cycles of plaquing on the restrictive host and grown to high titers in the corresponding SCC line. In this way, four presumptive *hr*Ad2 mutants were obtained: 0-12B2hrAd2 and 4-B2hrAd2 were selected in SCC12B2 and mutants 0-L1hrAd2 and 5-L1hrAd2 were selected in SCL1. The 0, 4, and 5 notations indicate the duration of nitrous acid mutagenesis of the original viral stock.

* Corresponding author.

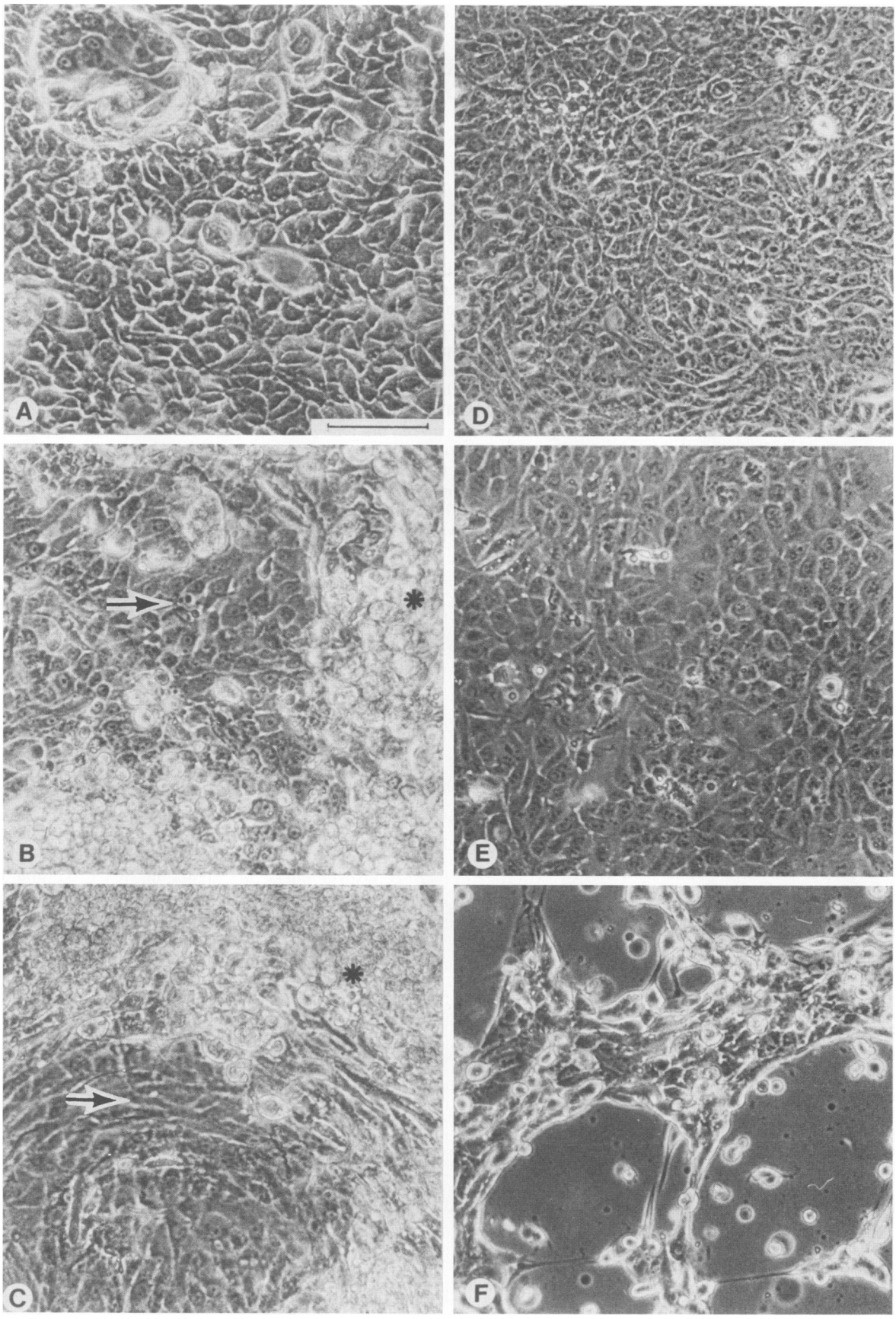
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TABLE 1. Properties of wtAd2 and hrAd2 infections in various keratinocyte hosts

Virus	Host	PFU/cell[a]	CPE[b]
wtAd2	NEKC[c]	10	±
	SCC12B2	29	−
	SCL1	126	−
	HeLa	531	+
0-12B2hrAd2	SCC12B2	595	+
	SCL1	519	+
	NEKC	560	±
	HeLa	572	+
4-12B2hrAd2	SCC12B2	610	+
	SCL1	521	+
	NEKC	585	+
	HeLa	592	+
0-L1hrAd2	SCL1	528	+
	SCC12B2	513	+
	NEKC	509	±
	HeLa	549	+
5-L1hrAd2	SCL1	583	+
	SCC12B2	529	+
	NEKC	538	±
	HeLa	583	+

[a] All infections were performed at a multiplicity of infection of 40 PFU per cell, and progeny virus production was measured at 3 days. Titers of wtAd2 and hrAd2 were determined on monolayers of HeLa cells (23).

[b] CPE is reported as + or − for monolayer cultures of SCC cells depending on whether or not a CPE was evident (Fig. 1). A notation of ± is used for cultures of stratified squamous epidermal keratinocytes to indicate CPE in suprabasal cells but not basal cells (Fig. 1).

[c] NEKC, Normal epidermal keratinocytes.

The properties of the hrAd2 mutants are listed in Table 1. Each of the four *hr*Ad2 mutants produced CPE and high yields of progeny virus 3 days p.i. in the SCC line used for mutant selection and in the other SCC line (range, 528 to 610 PFU per cell). When *hr*Ad2 mutants were passaged in nonrestrictive HeLa cells, the mutant phenotype persisted in SCC lines (data not shown). This indicates stability of the mutation. Cultures of epidermal keratinocytes from foreskin from newborns were initiated (20, 21, 25) and used in the second passage. When these cultures were infected at 40 PFU per cell with any of the four *hr*Ad2 mutants, high yields of progeny virus were produced (range, 509 to 585 PFU per cell; Table 1). This indicates that the *hr*Ad2 mutants had indeed overcome the restriction(s) to wtAd2 expression. However, like wtAd2, *hr*Ad2 mutants induced CPE in only the suprabasal cells (Fig. 1C). The basal cells remained normal in appearance. Thus, *hr*Ad2 mutants that are able to replicate to high levels in suprabasal epidermal keratinocytes cannot overcome the restriction present in basal cells. These results support the hypothesis that wtAd2 expression is restricted at different points in basal and suprabasal keratinocytes. The interactions of wtAd2 and *hr*Ad2 with cultured keratinocytes are illustrated graphically in Fig. 3.

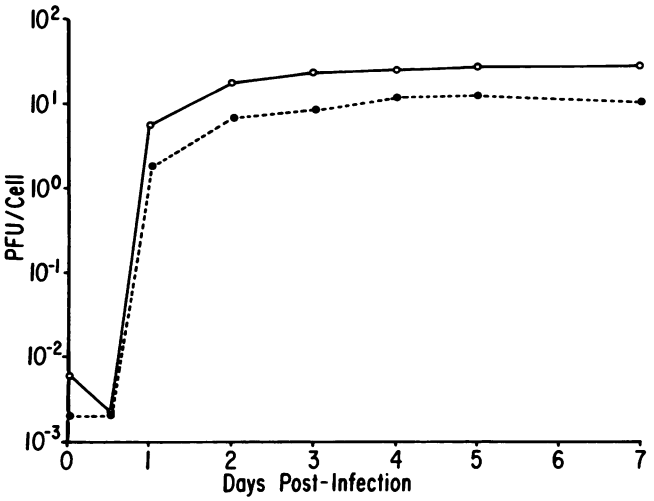

FIG. 2. Growth curve of wtAd2 in normal epidermal keratinocytes (---) and SCC12B2 (——). Normal epidermal keratinocyte and SCC12B2 cultures were infected with wtAd2 at a multiplicity of infection of 40 PFU per cell. At the times indicated, duplicate samples were lysed and virus titers were determined. Note that time zero is after a 90-min incubation to allow adsorption and internalization of virus.

Adenovirus replication in CV1 cells also appears to be restricted at multiple levels. In CV1, synthesis of early mRNA, early protein, and adenovirus DNA occurs as in permissive cells (10, 17, 22). However, late viral gene expression is restricted as noted by a 10-fold decrease in late viral mRNA and a parallel 10-fold decrease in late viral protein (7, 13). However, an additional restriction exists in the production of fiber protein. Fiber protein levels are reduced 100- to 1,000-fold, possibly the result of a specific defect in the processing of fiber mRNA (3, 4). It is unlikely, however, that the restrictions seen in CV1 cells are similar to either of the restrictions present in cultured epidermal keratinocytes. An *hr*Ad2 mutant capable of vegetative replication in CV1 cells ($Ad2^+$ND3 hr603 [2]) produced less than 1 PFU per cell in cultures of epidermal keratinocytes (data not shown). In addition, coinfection with Ad2 and simian virus 40, which results in vegetative replication of the Ad2 in CV1 cells (15, 16) does not have a similar effect in epidermal keratinocytes (data not shown).

It is possible that one or both points of restriction for Ad2 in epidermal keratinocytes are related to the epidermal origin of these cells. The epidermis is not a site typically infected by adenoviruses; in fact, adenoviruses usually infect the epithelium of the upper respiratory tract (5, 8). Keratinocytes cultured from oral-pharyngeal surfaces do support high-titer Ad2 replication, and the basal cells in these cultures do exhibit CPE (Aneskievich and Taichman, in press).

FIG. 1. Morphological responses of cultured keratinocytes to infection with wtAd2 and hrAd2. All cultures were infected with a multiplicity of infection of 40 PFU per cell and photographed at 3 days p.i. (A) Normal epidermal keratinocytes mock infected. These cultures are composed of multiple layers of cells. The cells in the upper layers are larger and often more refractile. Bar, 100 μm. (B) Normal epidermal keratinocytes infected with wtAd2. The basal cells (arrow) retain a normal appearance while the suprabasal cells exhibit CPE (*). (C) Normal epidermal keratinocytes infected with 0-12B2hrAd2. The response is the same as in panel B. Identical responses were obtained when 4-12B2hrAd2, 0-L1hrAd2, and 5-L1hrAd2 were used. (D) SCC12B2 cells mock infected. Note the monolayer organization of these cells in culture. SCL1 cells have a very similar appearance. (E) SCC12B2 cells infected with wtAd2. No CPE is evident. A similar result is seen when SCL1 cells are infected with wtAd2. (F) SCC12B2 cells infected with 0-12B2hrAd2. Note that all cells exhibit CPE. All hr mutants isolated induced this same response in SCL1 cells.

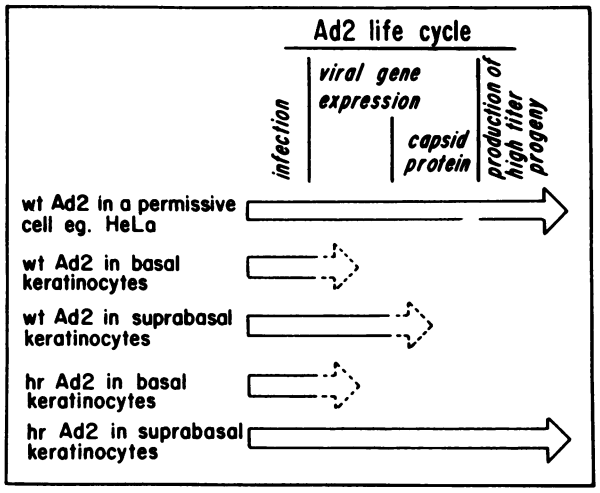

FIG. 3. Schematic illustration of the dual restriction to Ad2 expression in cultures of epidermal keratinocytes. The life cycle of Ad2 is illustrated along a horizontal line. In HeLa cells, which are fully permissive for Ad2, the line goes to completion. Restricted expression is indicated by a shortening of the arrow. Restriction early in the viral life cycle is noted by a greater shortening than a restriction later in the life cycle. The dotted arrow indicates a reduction in the level of Ad2 expression such as a reduction in progeny output.

Thus, restriction of Ad2 expression is a feature unique to epidermally derived keratinocytes and is not a property of mucosally derived cells. it is possible that one or both points of restriction in epidermal keratinocytes arise as a result of events that characterize these cells as epidermal.

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