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E1a-dependent expression of adenovirus genes in OTF963 embryonal carcinoma cells: Role of E1a-induced differentiation

(retinoic acid/cAMP/gene regulation)

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ABSTRACT **Some undifferentiated F9 embryonal carcinoma cells allow adenovirus genes to be expressed independently of the *E1a* oncogene normally required for their activation; this has been attributed to a cellular equivalent of E1a in F9 cells. However, transcription of all early genes was low in undifferentiated OTF963 embryonic carcinoma cells during the first 48 hr after infection with adenovirus type 5 (Ad5). Transcription then increased to about the level seen 16 hr after infection of cells induced to differentiate by retinoic acid (RA) (referred to as RA-dF9 cells), but this increase did not occur in cells infected by the *E1a* deletion mutant dl312. Addition of *E1a* in trans, or of RA, had no immediate effect on viral transcription in OTF963 cells, but viral transcription increased about 48 hr after these additions. Ad5 induced transcription of several differentiation-specific genes in OTF963 cells with about the same kinetics as their induction by RA. These genes were superinduced in RA-dF9 cells by cAMP or infection by adenovirus. We suggest the small amount of E1a produced early in infection of OTF963 cells activates cellular genes, some of which are differentiation specific and required for efficient transcription of viral genes, so that E1a both induces and is induced by differentiation. The simple hypothesis of a cellular equivalent to E1a does not adequately explain the complex interactions between viral and cellular genes in OTF963 embryonic carcinoma cells.**

Adenovirus early gene expression in permissive human cells is induced by the *E1a* oncogene, probably by E1a activation of a number of cellular transcription factors (1–3). E1a also induces several cellular genes, including *hsp70* (4), and it represses gene expression dependent on enhancers of papovaviruses (5). In some sublines of the embryonic carcinoma (EC) cell line F9, the adenovirus gene *E2a* is expressed at a high level independently of viral *E1a*, *hsp70* is constitutively expressed, and the papovavirus enhancers are repressed. In F9 cells induced to differentiate by retinoic acid (RA) and cAMP (RA/cAMP-dF9 cells), however, adenovirus *E2* expression is dependent on *E1a*, papovavirus enhancers are activated, and *hsp70* is not transcribed. These observations suggest that F9 EC cells contain an "E1a-like factor," which is lost on differentiation together with the malignant phenotype (4).

Some observations are not consistent with this simple hypothesis, if variability between F9 sublines (all of which have a common origin) is taken into account. In F9-41 cells, adenovirus early genes are not efficiently expressed (6). We have made similar observations in OTF963 cells, in which the defect in early adenovirus type 5 (Ad5) gene expression is relieved after a delay. Activation of the adenovirus *E2* gene by E1a in permissive cells is due, at least in part, to activation of transcription factor E2F, and E2F is activated in some F9 stem cells (7, 8). However, E2F has recently been shown to be activated by the viral *E4* gene in F9 cells, and (by a different mechanism) by *E1a* in differentiated cells (9); E2F is refractory to activation by *E1a* in undifferentiated F9 cells. The activity that allows E1a-independent transcription of *E2a* in some lines of F9 cells is therefore more "E4-like" than "E1a-like." But an "E4-like" factor does not explain the malignancy of F9 stem cells or the inactivity of papovavirus enhancers, which is likely due to an absence of some positively acting factors and the presence of some negatively acting factors, with an overall balance against efficient early gene expression (1, 10–13).

In this paper we describe experiments on adenovirus and cellular gene expression in OTF963 cells, F9 cells which lack factors that allow efficient expression of *E2* in the absence of viral E1a early in infection. The results suggest complex interactions between viral and cellular gene expression, in which the virus both influences and is influenced by the processes of differentiation.

MATERIALS AND METHODS

Cells and Viruses. OTF963 cells were obtained from M. Sleigh [Commonwealth Scientific and Industrial Research Organization (CSIRO), North Ryde, Sydney, Australia]. Growth of the cells, induction of differentiation by RA, and methods for infection by wild-type (wt) Ad5 and the *E1a* deletion mutant dl312 have been described (14, 21).

Plasmids and Transfection of F9 Cells. CMVE1a has the adenovirus *E1a* gene [nucleotides 356–1571 of adenovirus type 2 (Ad2)] driven by a cytomegalovirus promoter in a pBR322 background; a pBR322 control was used. F9 cells were transfected by a modified calcium phosphate precipitation method (15). Plasmids used as probes were as follows: pE1a, nucleotides 0–1571 of Ad5 in pBR322; pspE1b, nucleotides 1831–3931 of Ad2 in psp65; E2, nucleotides 21606–27372 of Ad5 in pBR322; E3, nucleotides 28962–32172 of Ad5 (X. Zhang); *E4*, nucleotides 32264–35938 of Ad2; *myc*, 12-kilobase (kb) *Eco*RI fragment of c-*myc* gene (16); α-tubulin, human cDNA 2.1-kb fragment in Puc19 (provided by N. J. Cowan, Princeton University); pUR-HS, *hsp70* (kindly provided by J. R. Nevins, Rockefeller University); EJ-*ras* (obtained from A. Greenberg, Manitoba Institute of Cell Biology); c-*src* (from M. Sleigh); laminin B2, a 700-base-pair (bp) fragment (provided by T. Lockett, CSIRO, Sydney) (11); c-*myb*, 2.1-kb mouse cDNA; and c-*src*, 0.8-kb *Pvu* II frag-

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Abbreviations: Ad5, adenovirus type 5; dl, deletion mutant; wt, wild type; iu, infectious units; RA, retinoic acid; RA-dF9, F9 cells induced to differentiate by 6 days of treatment with 0.5 μM RA; RA/cAMP-dF9, RA-dF9 cells also exposed to 1 mM N^6,$O^{2'}$-dibutyryl-cAMP during differentiation; CSIRO, Commonwealth Scientific and Industrial Research Organization.

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ment from the avian sarcoma virus (kindly provided by T. Lockett) (17).

Measurement of Transcription and mRNA Abundance. Transcriptional initiation was measured by nuclear run-on experiments (18), and mRNA abundance was measured by *in situ* hybridization on Zeta-Probe membranes (Bio-Rad) (19, 20). To quantitate results, a series of increasing autoradiographic exposures was scanned in a laser densitometer, and relative intensities of the signals were read from the linear region of the film response curve.

RESULTS

Kinetics of Relief of the Early Defect in Ad5 Transcription in OTF963 Cells. Quantitative comparison of transcription of early genes at different times after infection of OTF963 EC cells and RA-dF9 cells by 20 infectious units (iu) of Ad5 per cell showed that initiation of transcripts of all genes was one to two orders of magnitude less in undifferentiated cells for about 40 hr after infection (Fig. 1). Nevertheless, transcription of all early genes was just detectable between 20 and 40 hr after infection of F9 stem cells. *E2* and *E3* were expressed at a higher rate than the other genes early after infection of undifferentiated cells (Fig. 1). Although the higher expression of *E2* could be due to activation of E2F by a cellular "E1a-like" or "E4-like" factor, this does not explain the expression of *E3*, whose promoter does not contain an E2F binding site (3). Moreover, E2 transcription was almost two orders of magnitude less in OTF963 cells than RA-dF9 cells up to 20 hr after infection.

From 48 hr on, Ad5 transcription in OTF963 cells accelerated, to reach rates similar to those 16–24 hr after infection of RA-dF9 cells (Fig. 1). *E2* and *E3* were again transcribed at higher rates than the other genes, suggesting the presence of factors acting on these adjacent promoters, but this does not explain how from 48 hr after infection transcription of all Ad5 genes accelerated at about the same rate.

Role of RA-Induced Differentiation. Adenovirus early gene transcription began earlier in RA-dF9 cells, and it rose more rapidly and to a higher rate than in stem cells (Fig. 1*b*). RA added to the medium of stem cells, or withdrawn from the medium of RA-dF9 cells, had no effect on Ad5 early gene expression measured 16 hr later; the difference in viral gene expression between F9 and RA-dF9 cells is therefore due to RA-induced differentiation and not to a direct effect of RA on transcription. To determine at what time in the differentiation pathway RA activated adenovirus transcription, F9 cells were cultured in RA for 0–6 days and infected by Ad5, and the abundance of viral *E1a*, *E1b*, and *E3* mRNA was determined, with α-tubulin expression as a control. All the viral genes were transcriptionally activated on the third day of RA treatment to a level close to the maximum plateau of adenovirus expression in differentiated F9 cells (not shown).

E1a Activates Viral Gene Expression in F9 Cells After a Delay. Previously we suggested that the increase in Ad5 transcription that begins about 40 hr after infection of OTF963 cells depends on prior expression of viral *E1a* (21). To confirm this, and to test if low-level expression of adenovirus genes other than *E1a* could cause, in the absence of E1a, the changes in F9 cells necessary for efficient adenovirus transcription, F9 and RA-dF9 cells were infected with the *E1a*-mutant dl312 for 72 hr prior to superinfection with wt Ad5 and compared with cells infected by wt Ad5 alone. After 24 and 48 hr of wt superinfection, *E1a* and *E2a* mRNA abundance was determined. There was no increase in viral gene expression in cells preinfected with dl312 (Fig. 2). It was also apparent, contrary to reports with other F9 cells (4, 22), that dl312 was defective for transcription of *E2a* in OTF963 cells even after 72 hr of infection, and a similar result was obtained previously at high multiplicity of infection (21). This indicates that the activation of adenovirus gene expression in OTF963 cells is mediated by viral E1a in a time-dependent manner, and not by a cellular E1a-like factor present at the time of infection or another viral gene transcribed in an E1a-independent manner.

If lack of *E1a* transcription is solely responsible for the defect in viral gene transcription in OTF963 cells, then *E1a* added in trans should rapidly increase transcription of E1a-dependent genes. To test this, F9 and RA-dF9 cells were transfected by the *E1a* expression vector CMVE1a or with a pBR322 control. After 18 hr, the cells were infected by Ad5 or dl312 (20 iu per cell) for 24 hr, and assayed for *E1a* and *E2a*

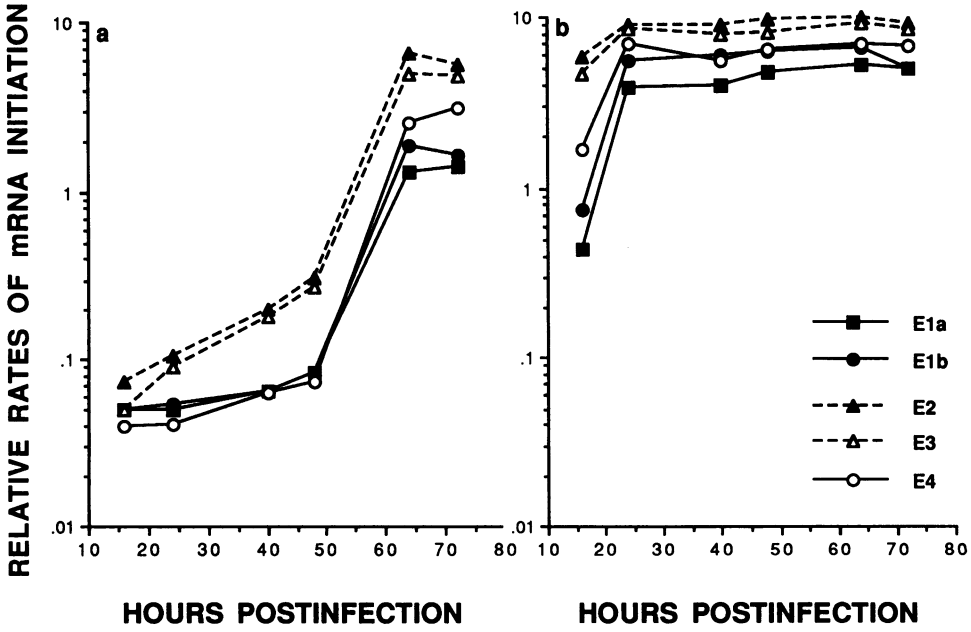

FIG. 1. Adenovirus transcription in OTF963 cells (*a*) and RA-dOTF963 cells (*b*). Cells were infected by 20 iu of Ad5 per cell, and at the times indicated, initiation of transcription of viral early genes was determined by nuclear run-on. The relative intensity of autoradiographic signals was determined by densitometry and normalized for slight variations in transcription of α-tubulin.

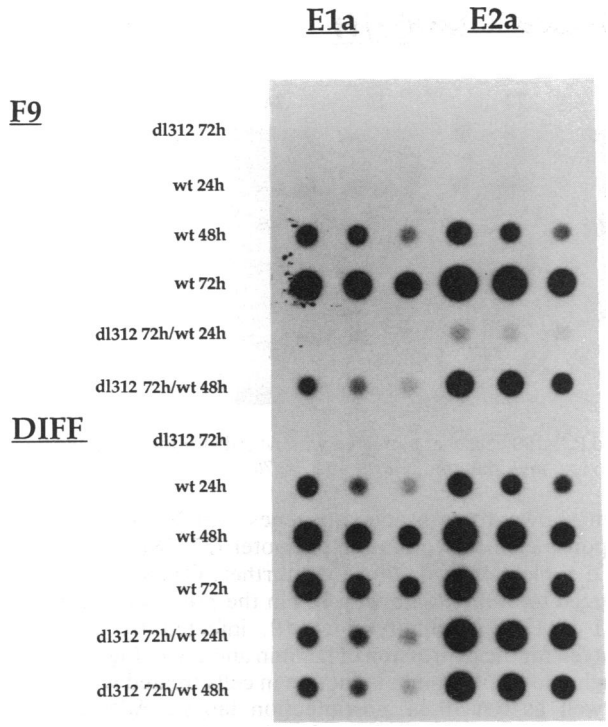

FIG. 2. Ad5 gene expression in OTF963 is E1a dependent. F9 and RA-dF9 (DIFF) cells were infected with (*i*) dl312 for 72 hr at 20 iu per cell; (*ii*) wt for 24, 48, or 72 hr; or (*iii*) dl312 for 72 hr and superinfected with wt for 24 or 48 hr prior to being assayed by *in situ* hybridization for *E1a* and *E2a* mRNA.

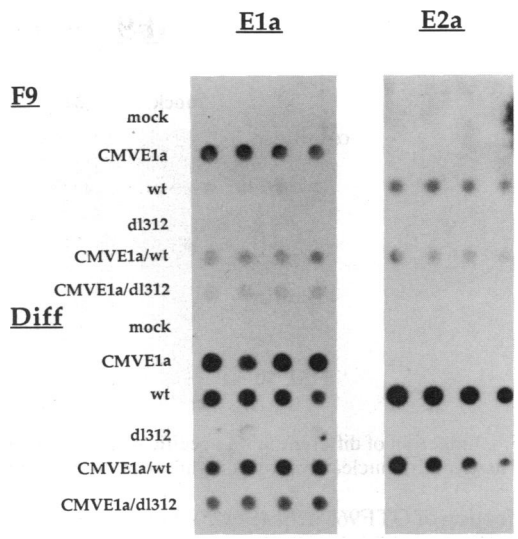

FIG. 3. Expression of CMVE1a at the time of infection does not affect the level of adenovirus transcription in OTF963 cells. F9 and RA-dF9 (Diff) cells were transfected with either CMVE1a or pBR322 DNA by calcium phosphate precipitation 18 hr prior to a 24-hr infection with either dl312 or wt Ad5 and assayed by *in situ* hybridization for *E1a* or *E2a* mRNA.

transcripts. *E1a* was transcribed from the CMVE1a promoter in both F9 and RA-dF9 cells (Fig. 3). However, this additional *E1a* transcription did not increase the *E2* transcription from wt virus, nor did CMVE1a detectably complement dl312. Thus *E1a* did not alter the level of viral transcription when added in trans shortly before infection. However, if E1a activated cellular genes required for activation of other adenovirus genes in OTF963 cells, then time as well as the amount of E1a might be a critical factor. To test this, CMVE1a was transfected 48 hr prior to a 24-hr infection with wt Ad5 or dl312. The cells were assayed by *in situ* hybridization for *E1a*, *E2a*, *E4*, and α-tubulin gene expression. Prior transfection with CMVE1a increased Ad5 gene expression in F9 cells about 6-fold (Fig. 4) but had no detectable effect in RA-dF9 cells. The activation of adenovirus gene expression was specific, as the levels of α-tubulin mRNA remained unchanged. *E1a* transcripts from the CMVE1a plasmid were no longer present in F9 cells at the time of assay. In RA-dF9 cells a small amount of *E1a* transcription was detectable from the CMVE1a plasmid and was able to transactivate *E2a* (Fig. 4). In undifferentiated F9 cells, prior transfection with CMVE1a increased transcription of *E1a*, *E2a*, and *E4* in wt-infected cells but was unable to activate *E2* or *E4* to the same level in dl312-infected cells. This suggests that activation of adenovirus early genes in F9 cells depends both on the level of E1a and on time-dependent activation of cellular genes by E1a.

Does Adenovirus Induce Differentiation of F9 Cells During Infection? The above results and the recent reports of *E1a*-induced differentiation of F9 cells (23–25) led us to hypothesize that the small amount of *E1a* expression during the initial phase of adenovirus infection of OTF963 cells might induce changes in transcriptional regulation similar to those that occur during RA-induced differentiation and thus result in efficient viral transcription late in infection. To test for *E1a*-mediated differentiation-specific events, OTF963 and RA-dF9 cells were infected with Ad5, and transcription of several differentiation-specific genes was measured by nuclear run-on. The differentiation-specific markers, laminin, *src*, *ras*, and *myc*, were transcriptionally activated 64–72 hr

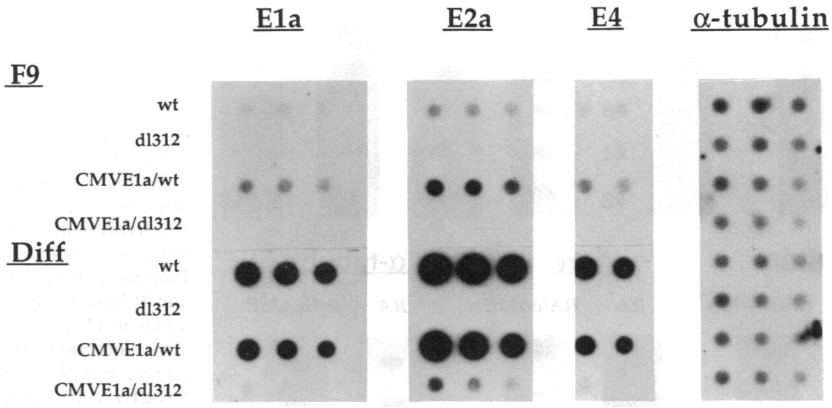

FIG. 4. Expression of CMVE1a 48 hr prior to infection increases adenovirus transcription in F9 cells. OTF963 (F9) and RA-dOTF963 (Diff) cells were transfected with either CMVE1a or pBR322 DNA 48 hr prior to a 24-hr infection with either dl312 or wt Ad5. Cells were then assayed by *in situ* hybridization for *E1a*, *E2a*, *E4*, and α-tubulin mRNA.

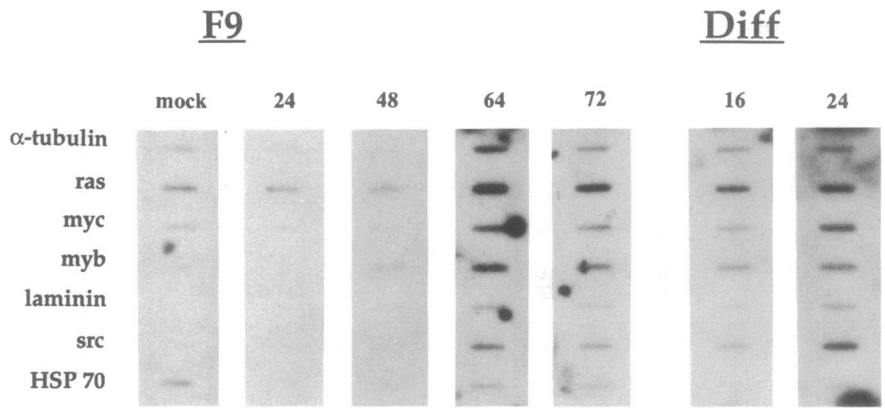

FIG. 5. Induction of differentiation-specific genes in OTF963 by Ad5. F9 and RA-dF9 cells were infected with wt Ad5 for the indicated times (hr) and assayed by nuclear run-on for transcription of α-tubulin, EJ-*ras*, c-*myc*, c-*myb*, laminin, c-*src*, and *hsp70*.

after infection of OTF963 cells (Fig. 5), but so was *myb*. *hsp70* transcription was high in OTF963 cells, as reported for other F9 cells (4), and it initially decreased but increased slightly during late infection to about the level in infected RA-dF9 cells. To investigate further the regulation of a differentiation-specific gene during adenovirus infection of F9 and RA-dF9 cells, cytoplasmic RNA samples were assayed for laminin mRNA abundance. There was a small but reproducible increase in laminin mRNA in OTF963 cells during the course of infection, to slightly above the level in uninfected RA-dF9 cells (Fig. 6). This confirmed that Ad5 induces some differentiation-specific events in F9 cells.

Superinduction of Cellular Genes by Ad5 or cAMP in RA-dF9 Cells. Laminin transcription was induced by adenovirus infection to a higher level in RA-dF9 cells (Fig. 6) than in undifferentiated cells, as was expression of c-*myc*, c-*myb*, and c-*src*. This was not a general increase in mRNA levels, as α-tubulin transcription did not increase. Laminin is transcriptionally activated by RA-induced differentiation and is activated further by cAMP (26). The activation of laminin by Ad5 in RA-dF9 cells suggests that Ad5 may replace the functional requirement for cAMP to induce this further step in differentiation. The *E1a* gene of adenovirus has previously been shown to transactivate genes which have a cAMP response element within the promoter (27, 28).

To explore the role of cAMP further, OTF963 cells were induced to differentiate with RA in the presence or absence of 1 mM $N^6,O^{2'}$-dibutyryl-cAMP, infected by Ad5, and assayed for the expression of laminin and c-*src*. Fig. 6*c* shows a higher level of laminin induction in cells treated with cAMP as well as RA (0 hr postinfection lanes). Ad5 infection increased the transcription of laminin in RA-dF9 cells to levels similar to those in RA/cAMP-dF9 cells but did not further induce laminin in the latter cells. The transcription of c-*src*, on the other hand, did not change in response to cAMP (17) and was superinduced by Ad5 in OTF963 cells induced to differentiate by RA with or without cAMP (Fig. 6*c*). Moreover Ad5, unlike cAMP, can induce differentiation-specific changes in the absence of RA. Thus a simple hypothesis of E1a replacing either RA or cAMP in differentiation events does not explain all of the observed effects.

DISCUSSION

Our results with OTF963 cells are consistent with those for F941 cells (6), in which viral *E1a* is expressed at a low level early in infection, and other E1a-dependent viral genes are

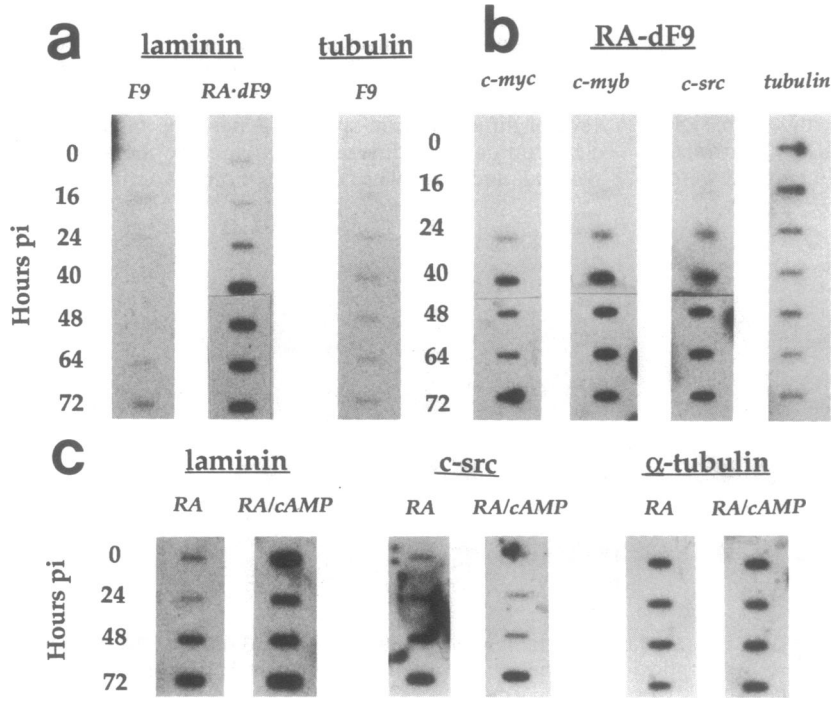

FIG. 6. Superinduction of cellular differentiation-specific genes by Ad5 and cAMP in RA-dOTF963 cells. F9, RA-dF9, and RA/cAMP-dF9 cells were infected with 20 iu of Ad5 per cell. At the indicated hr postinfection (pi) cytoplasmic RNA was isolated and assayed by *in situ* hybridization for expression of laminin, c-*myc*, c-*myb*, c-*src*, or α-tubulin. (*a*) Laminin cytoplasmic mRNA levels in F9 and RA-dF9 cells and α-tubulin mRNA levels in F9 cells as a function of adenovirus infection. (*b*) The expression of c-*myc*, c-*myb*, c-*src*, and α-tubulin in RA-dF9 cells during adenovirus infection. (*c*) Comparison between RA-dF9 and RA/cAMP-dF9 cells for the expression of laminin, c-*src*, and α-tubulin mRNA during adenovirus infection.

not efficiently activated by cellular factors. The discrepancy between these results and reports of a cellular "E1a-like" factor in F9 cells (4, 9) may be due to differences between cell lines. The low level of expression of c-*myc* in OTF963 cells (17, 29) compared with another F9 line (30) may be relevant; c-*myc* has been reported to activate adenovirus *E2* and *E4* (16, 31).

In undifferentiated OTF963 cells adenovirus transcription is relatively inactive during the first 48 hr of infection, but it then increases to a level similar to that in RA-dF9 cells. This early defect in OTF963 cells is not overcome by high-multiplicity Ad5 infection (21). We show in this paper that the increase in viral transcription depends on the presence of a functional *E1a* gene and that there is a significant increase in viral gene expression during early infection when undifferentiated cells are exposed to RA, or to E1a expressed from a promoter active in F9 cells 48 hr before infection.

These observations led to the hypothesis that the small amount of viral E1a produced early during infection of OTF963 cells mediates events, at least some of which are differentiation-specific, that result in activation of adenovirus genes. Supporting this hypothesis, the differentiation-specific markers laminin and c-*src* were shown to be transcriptionally activated in parallel with the activation of viral gene expression. Furthermore, an *E1a* plasmid caused localized areas of F9 cells to undergo morphological changes reminiscent of differentiation (data not shown). Our results are consistent with recent reports that E1a induces differentiation of other F9 cells (23–25).

It has recently been reported that the expression of *E1a* causes retrodifferentiation of differentiated F9 cells, as determined by a reduction in the transcription of collagen type IV and plasminogen activator mRNA during adenovirus infection (22). Our results, however, showed that transcription of laminin and c-*src* mRNA is superinduced by Ad5 in RA-dOTF963 cells. The data on superinduction of laminin suggest that adenovirus may mimic cAMP in further activation of laminin in RA/cAMP-dF9 cells. RA and cAMP act in synergy to differentiate F9 cells from a primitive endoderm into parietal or visceral endoderm (26, 32). cAMP activates a network of genes which contain cAMP response elements in their promoters (33); similar elements are found in adenovirus promoters (28, 34, 35) and may be activated by cAMP and E1a in synergy (27). It has been suggested that E1a may be able to functionally substitute for cAMP in F9 cells for the activation of some genes (27, 28). In our experiments, however, the expression of c-*src* was not influenced by cAMP in RA-dF9 cells but was still superinduced by adenovirus infection.

Although our results suggest that many of the changes induced by E1a in OTF963 cells are differentiation specific, others are not. Moreover, the data are inconsistent with hypotheses that E1a simply acts like RA, cAMP, or a combination of both. We likewise believe our data cannot be explained by "E1a-like" and/or "E4-like" factor(s) in OTF963 stem cells. In F9 cells, adenovirus early genes have complex time-dependent regulatory interactions with each other, and with networks of cellular genes, all of which are influenced by the genetic and epigenetic history of the cell and environmental signals to which it is, and has been, exposed. In OTF963 cells, E1a appears to both induce and be induced by differentiation in a way that suggests nonlinear positive feedback that also responds to RA and cAMP. It may be impossible to completely understand complex changes such as differentiation and malignant transformation by a molecular genetic approach, because it is essentially static. Rather it may be necessary to develop a systems approach, in which gene regulation is modeled as a complex set of dynamic interactions.

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