Defective Expression of Adenovirus Genes during Early Infection of Undifferentiated OTF963 Embryonal Carcinoma Cells

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Adenovirus infection was compared in F9 (OTF963) cells and cells induced to differentiate with retinoic acid, in order to study expression of early genes under the control of the reported "E1a-like factor" in F9 cells. However, not only was transcription of the viral E1a gene defective in undifferentiated cells but expression of all the other early genes was found to be reduced in OTF963 cells in comparison to differentiated cells. The defect in early gene expression was detected at the level of transcriptional initiation during the first 48 h of infection and resulted in similarly low levels of viral cytoplasmic mRNA and viral protein synthesis. Viral DNA replication was delayed and reduced. After 48 h of infection, the defect in transcription in OTF963 cells of E1a and other early genes was relieved, so that by 72 h postinfection the level of transcription was similar to that 16 h after infection of differentiated cells. At no time did adenovirus early gene expression occur independently of viral E1a. These results suggest limits to the generality and explanatory power of the hypothesis that F9 embryonal carcinoma cells contain an E1a-like factor.

When embryonal carcinoma (EC) cells differentiate, radical changes in their morphology (44), proliferative rate (25, 38, 40), and tumorigenicity (45) are observed. F9 cells do not undergo spontaneous differentiation under normal culture conditions but can be induced to differentiate in vitro in the presence of retinoic acid into primitive endoderm-like cells (44), like those of the inner cell mass of the blastocyst (17). F9 cells treated with retinoic acid and dibutyryl cyclic AMP differentiate further to form either parietal or visceral endoderm (14, 46).

EC cell lines have provided a useful in vitro model for the study of changes in gene regulation during differentiation (28). Genes encoding laminin B, collagen type IV, major histocompatibility complex class I antigens, β -2 microglobulin, Endo A, Endo B, and vinculin are transcriptionally activated 3 days postinduction. The cellular proto-oncogenes *c-abl*, *c-src*, and *c-fos* are also transcriptionally activated 1, 4, and 6 days postinduction of differentiation, respectively (28, 40).

Viral genes have been used as a model system to investigate changes to gene regulation as a function of differentiation because they use cellular transcription factors and thus may reflect the regulation of cellular gene transcription. It has been found that transcription of several viral genes is repressed in undifferentiated F9 cells. However, if F9 cells are induced to differentiate, viral genes are expressed efficiently. The enhancer elements in the long terminal repeats of retroviruses, Moloney murine leukemia virus, murine sarcoma virus, and Rous sarcoma virus appear to be under negative control in undifferentiated F9 cells (10, 12, 13, 27, 49). The DNA viruses simian virus 40 (SV40), polyomavirus, and cytomegalovirus are all inactive in F9 cells due to a defect in the function of their early gene enhancers (11, 12, 32, 41, 42, 47, 48, 50). Since the immediate-early gene products of these viruses are not produced, transactivation

of the other viral genes does not occur and the infection becomes abortive.

F9 cells have been reported to be permissive for adenovirus infection on the basis of the results of Imperiale et al. (18), which showed similar levels of E2a protein in F9 and differentiated F9 cells after adenovirus infection. In this study, it was reported that F9 cells have an elevated level of heat shock protein 70 (hsp70) which rapidly decreases upon induction of differentiation. hsp70 transcription can be activated by many stimuli, including the E1a gene products (21). When F9 cells were infected with the E1a⁻ mutant *d1312* (4, 19, 31, 39), they were reported to express E2a protein, whereas E2a expression was undetectable in differentiated F9 cells infected with *d1312* (18). These observations led to the hypothesis that F9 cells contain a cellular "E1a-like factor," which disappears as a function of differentiation (18). F9 cells have since been shown to contain an activated form of the cellular transcription factor E2F which is necessary for efficient transcription of E2a (35). The inactive form of E2F in differentiated F9 cells is activated by phosphorylation during a wild-type adenovirus infection in an E1a-dependent manner (2), which requires the E4 as well as E1a gene products (1, 36).

Since the initial report of an E1a-like factor in F9 cells, other researchers have attributed numerous characteristics of F9 cells to the E1a-like factor because of the similarity of F9 characteristics with the reported effects caused by E1a in other cell types, for instance, the oncogenic state of the cell (53). The repression of the SV40 and polyomavirus early enhancers in F9 cells is often attributed to the presence of an E1a-like factor (13, 24, 42, 43). This argument is based on findings obtained with HeLa cells, for which it was reported that SV40 (51) and polyomavirus early enhancers are repressed by the E1a gene products (5). It was also shown that E1a gene products could repress the E1a enhancer (5).

In this paper, we report experiments on adenovirus infection of one of the F9 cell subclones commonly used, OTF963 (37). We initially expected that the viral E1a gene might be transcriptionally repressed via its enhancer but that other early genes would be activated by the E1a-like factor re-

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ported in F9 cells (18). However, we found that transcription of all adenovirus genes, not just E1a, was initially defective in OTF963 cells. At later times, these defects in adenovirus transcription were relieved. These results led us to question the universality and explanatory power of the hypothesis of an E1a-like factor in F9 EC cells.

MATERIALS AND METHODS

Viruses and cells. The F9 cells used in this study, OTF963 (37), were obtained from M. Sleight (CSIRO, Sydney, Australia) and were maintained on gelatin-coated flasks in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum at 37°C in a humidified 6.8% CO₂ incubator. Differentiation of the OTF963 cells was initiated by the addition of 5×10^{-7} M all-*trans* retinoic acid to the medium, and cells used 6 days postinduction are referred to as differentiated. Adenovirus type 5 (Ad5) and the E1A mutant *dl312* were propagated and titrated as described elsewhere (3), except that OTF963 cells were infected in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

In situ hybridization on membranes. In situ hybridization was carried out by the method of Paeratakul et al. (34). Cells were harvested by trypsinization, pelleted, and suspended in phosphate-buffered saline at a concentration of 5×10^5 cells per ml. A Bio-Rad Hybridot apparatus was assembled by using a Zeta Probe hybridization membrane prewetted in phosphate-buffered saline. A series of twofold dilutions was made by dispensing 200, 100, or 50 µl of cells in adjacent wells, equivalent to 1×10^5 , 5×10^4 , and 2.5×10^4 cells per well, respectively. To detect viral DNA, the cells were fixed to the Zeta Probe membrane and lysed by the addition of 300 µl of 0.5 M NaOH to each well. After vacuum filtration, the filter was air dried. The filter was prewet in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) immediately prior to prehybridization at 65°C for more than 2 h in 10 ml of the following: 1.5× SSPE (1× SSPE is 0.18 M NaCl–10 mM Na₂HPO₄–1 mM EDTA), 1% sodium dodecyl sulfate (SDS), 0.5% Blotto (10% nonfat dry milk), and 0.5 mg of sheared and denatured DNA per ml. Filters were hybridized to specific probes in prehybridization buffer for 16 to 24 h. The probes were labeled by the random priming method.

To detect RNA, the cells were fixed for 1 h at 4°C in 100 µl of 1% glutaraldehyde–3% NaCl–10 mM NaH₂PO₄–40 mM Na₂HPO₄ per well. The fixed cells were washed three times with 100 mM Tris (pH 8.0)–50 mM EDTA in the apparatus with a vacuum applied. The cells on the filter were digested at 37°C for 30 min in 20 ml of 100 mM Tris (pH 8.0)–50 mM EDTA containing 40 µg of proteinase K per ml. Filters were air dried and rinsed in 2× SSC and prehybridized at 43°C for more than 2 h in 50% deionized formamide–2× SSPE–1% SDS–0.5% Blotto–0.5 mg of herring testis DNA per ml. Filters were hybridized in prehybridization buffer containing $>10^7$ cpm of the randomly primed labeled specific probe at 43°C for 16 to 24 h.

Both DNA and RNA filters were washed after hybridization at room temperature for 15 min in 2× SSC–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS. Damp filters were sealed in bags and autoradiographed.

Nuclear run-on assays. Nuclear run-on transcription assays were performed as described by Linial et al. (26). Zeta Probe membrane filters contained 5 µg of alkaline-denatured plasmid DNA per slot and were prehybridized at 43°C for 16 h in 50% formamide–5× SSPE–1 mM EDTA–5× Denhardt solution–0.5 mg of herring testis DNA per ml–0.5% SDS.

Run-on products were suspended in hybridization buffer (50% formamide, 5× SSPE, 1 mM EDTA, 2× Denhardt, 0.5 mg of herring testis DNA per ml, 0.5% SDS) and hybridized to filters for 72 h at 43°C. Filters were washed twice at room temperature for 15 min in 2× SSC–0.1% SDS and twice at 55°C for 20 min in 0.2× SSC–0.1% SDS. Filters were digested with 10 µg of RNase A per ml in 2× SSC at 37°C for 30 min and rinsed in 2× SSC–0.1% SDS at room temperature.

Immunoprecipitation. The following antibodies were used: for E1a, monoclonal antibody M73 (generously supplied by E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); for the E1b 58-kilodalton (kDa) protein, polyclonal serum H14b from tumor-bearing hamsters (generously supplied by E. Blair, Leeds University, Leeds, United Kingdom); for hexon, a polyclonal serum (E. Blair); and for p53, polyclonal serum pAb 200-47 (6). Immunoprecipitation of proteins was performed by the method of Braithwaite and Jenkins (6) as follows. Cells were infected with wild-type Ad5 at a multiplicity of infection (MOI) of 20 infectious units (iu) per cell and at the indicated times were incubated in medium containing 150 µCi of [³⁵S]methionine for 1.5 h at 37°C. Cells were lysed in RIPA buffer (10 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate) containing 20 µl of aprotinin. The lysate was centrifuged at 120,000 × *g* for 30 min at 4°C to remove cellular debris and was precleared with protein A-Sepharose CL-4B beads prior to immunoprecipitation with the specific antibody. Samples were subjected to SDS-polyacrylamide gel electrophoresis on 9% polyacrylamide gels. Gels were fixed in 40% methanol–10% acetic acid, enhanced with Amplify (Amersham Corp.), dried, and exposed to autoradiographic film.

Plasmids. pBR322-derived plasmids containing the following inserts were used as probes on the filters: for E1a, nucleotides 0 to 1,571 of Ad5; for E1b, nucleotides 1,831 to 3,931 of Ad2; for E2, nucleotides 21,606 to 27,372 of Ad5; for E3, nucleotides 28,962 to 32,172 of Ad2; for E4, nucleotides 32,264 to 35,938 of Ad2; and for late genes, nucleotides 9,689 to 19,331 of Ad2.

RESULTS

Adenovirus early genes are poorly expressed in OTF963 cells. Our initial objective was to test for E1a transcriptional repression relative to other viral genes during a wild-type adenovirus infection in OTF963 cells in comparison to differentiated cells. Viral mRNA abundance in OTF963 and differentiated OTF963 cells was compared by hybridization of DNA probes representing all the early gene regions (Materials and Methods). The results showed that not only was transcription of E1a severely reduced in OTF963 cells but transcripts of the other early regions were also reduced compared with those in differentiated cells during the first 48 h after infection (Fig. 1). However, by 48 h postinfection, transcription of all adenovirus genes was detected in OTF963 cells, at which time viral transcripts in OTF963 cells were approximately at the same level as viral transcripts in differentiated OTF963 cells at 16 h postinfection. The specificity of the defective transcription of adenovirus genes was shown by the hybridization signal of α-tubulin mRNA, which did not vary significantly with differentiation or infection.

The relative amounts of viral transcripts from a number of duplicate experiments were quantitated by densitometry of autoradiograms of filters hybridized to the indicated probes,

FIG. 1. In situ hybridization of adenovirus mRNAs in OTF963 and differentiated OTF963 cells. OTF963 and differentiated OTF963 cells were infected at a multiplicity of 20 iu per cell, and at the indicated hours after infection (shown at the left of the gels), the cells were harvested and serial twofold dilutions were loaded onto a Hybri-dot filtration apparatus containing a Zeta Probe hybridization membrane. Cells were fixed to the membranes and digested with proteinase K. Specific mRNAs were detected with radiolabeled probes for various adenovirus genes and α -tubulin.

and the average results of the experiments were plotted against time. Alternatively hybridized signals were quantitated by scintillation counting of the dots. In all cases, the results paralleled those obtained by densitometry. The most prominent feature of the viral transcriptional profile for all genes (Fig. 2) was an initial period of severely reduced adenovirus gene expression in OTF963 cells. However, viral transcription in OTF963 cells then increased until at 48 h after infection it was at a level similar to that 16 h after infection of differentiated OTF963 cells. The lack of adenovirus gene expression during the early phase of infection parallels that found in SV40 and polyomavirus infections of F9 cells, but no viral transcription is seen even after 14 days of papovavirus infection (50).

To ensure that the in situ hybridization technique detected only mRNA and not viral DNA, duplicate filters were treated with RNase prior to hybridization and showed no hybridization. Cytoplasmic RNA samples from infected OTF963 and differentiated cells were analyzed by Northern (RNA) blot to ensure that the in situ hybridization technique accurately represented mRNA of the expected size class. The E1a transcripts were of the previously reported size and showed the same relative transcriptional pattern as that detected by in situ hybridization.

A conservative estimate of the reduction in adenovirus gene expression in OTF963 cells compared with their differentiated counterparts at 16 h postinfection ranged from ~15-fold for E2 and E3 to ~35-fold for E1a and E1b to at least 40-fold for E4 and late gene syntheses, which were barely detectable. At 24 h postinfection, the difference in viral transcripts increased, ranging from 30- to 100-fold for E2 and E4, respectively. After 48 h of infection, all viral genes in OTF963 cells began to be expressed and the difference between expression in undifferentiated and differentiated cells decreased.

If there is an E1a-like factor in OTF963 cells, it is not equivalent to viral E1a. The data presented above showed that all viral gene transcripts were present at lower levels in OTF963 cells when compared with differentiated OTF963 cells. Imperiale et al. (18) reported that in the complete absence of E1a there was a significant amount of transcription from the E2a promoter in F9 cells. However, a direct comparison of the efficiency of the E1a-like factor and viral E1a for the transactivation of E2a in F9 cells was not made. Our results showed that in OTF963 cells infected with wild-type adenovirus, the E2 and E3 genes were expressed more (approximately 2-fold) than E1a, E1b, and E4, but

FIG. 2. Quantitation of relative abundance of adenovirus mRNAs in OTF963 and differentiated OTF963 cells. Adenovirus transcripts were determined in several independent in situ hybridization experiments of the type shown in Fig. 1, quantitated by densitometry, averaged, and plotted against time. ■, Differentiated OTF963 cells; □, undifferentiated OTF963 cells.

expression of E2 and E3 was still substantially reduced (15- to 30-fold) in comparison with differentiated OTF963 cells.

To directly compare the efficiency of the putative cellular E1a-like factor in OTF963 cells and viral E1a for the transactivation of adenovirus early genes, OTF963 cells were infected with wild-type Ad5 or *dl312* at increasing multiplicities, which ranged from 5 to 1,000 iu per cell. The cells were assayed for E1a and E2a gene expression at 48 h after infection by in situ hybridization. The amount of E2a transcription in OTF963 cells infected with *dl312* at a multiplicity of 1,000 iu per cell after 48 h infection was approximately 20- to 30-fold less than that in wild-type infected cells (Fig. 3). This indicates that if there is an OTF963 E1a-like factor, it is more than an order of magnitude less effective than the viral E1a gene for the transactivation of E2a, even under conditions where the viral E1a gene is itself repressed and not functioning optimally.

Defective expression of adenovirus genes in OTF963 cells cannot be overcome by high MOI. It has been suggested that high template numbers of SV40 DNAs following transfection of F9 cells can overcome transcriptional repression, presumably by titrating out a negative factor (13). If the difference in adenovirus mRNA abundance between OTF963 EC cells and differentiated cells were due solely or mainly to a repressor of transcription in F9 cells which was lost upon differentiation, perhaps the repressor could be titrated out by a high MOI. To test this hypothesis, OTF963 and differentiated cells were infected with wild-type virus at multiplicities from 5 to 1,000 iu per cell and compared for levels of viral mRNA. The results showed that relatively high levels

of multiplicity did not detectably affect the level of viral mRNA in OTF963 cells (Fig. 4). We have interpreted this to mean either that (i) a repressor is present in OTF963 cells but it is present at a level much higher than 1,000 copies per cell or (ii) the lack of viral transcription is not due to the presence of a repressor.

On a duplicate filter, viral DNA levels in OTF963 cells were assayed by in situ hybridization. The results showed that viral DNA template numbers increased, as expected,

FIG. 3. Comparison of E1a and E2a transcription in wild-type (WT)- and *dl312*-infected OTF963 cells. OTF963 cells were infected by Ad5 WT or *dl312* at the indicated multiplicities. Cells were assayed for E1a and E2a mRNAs 48 h postinfection by in situ hybridization, as described in the legend to Fig. 1.

FIG. 4. High-multiplicity Ad5 infection does not overcome the early defect in OTF963 cells. Cells were infected at the indicated multiplicity, and E1a and E2a mRNA abundance was estimated 24 h later, as described in the legend to Fig. 1.

with an increasing MOI (Fig. 4). Viral mRNA, however, reached a plateau of expression at a relatively low level of multiplicity (5 to 50 iu per cell) in both undifferentiated and differentiated OTF963 cells. Thus, viral mRNA levels were not influenced by additional viral templates in OTF963 or differentiated OTF963 cells. This suggests that the amount of viral transcription that can occur at a given time is limited by positive cellular factors in both cell types and that the amount of such factors is higher in differentiated OTF963 cells.

Defective viral transcription in OTF963 cells is not due to reduced template numbers. Adenovirus particles are found near the nuclear membrane by 20 min postinfection; the viral core is then uncoated and passes through a nuclear pore into the nucleus (for a review, see reference 30). When viral gene regulation is studied, such processes are often not taken into consideration. However, since we were examining adenovirus infection in two different cell types, we felt it was necessary to ensure that differences in viral mRNA levels were not due to differences in viral adsorption, penetration, and transport of viral DNA into the nucleus, which could affect the number of viral DNA templates available for transcription.

To determine whether adenovirus was able to adsorb to and penetrate cells and to enter the nuclei of OTF963 and differentiated OTF963 cells with equal efficiency, cells were infected with ^3H -labeled adenovirus and the amount of total and acid-insoluble ^3H -labeled viral DNA was determined in washed cells and nuclei 4 h later. From the results of these experiments, it was evident that reduced adsorption, transfer of viral DNA to the nucleus, and stability of viral DNA could account at most for two- to fourfold less viral DNA in adenovirus-infected OTF963 cells compared with their differentiated derivatives (data not shown). Since multiplicity did not significantly affect the levels of mRNA transcription, it seems unlikely that these slight differences in DNA template numbers could account for the 30- to 100-fold differences in the levels of viral transcription observed at early times of infection.

Lack of viral mRNA in OTF963 cells is due to reduced transcriptional initiation. In order to determine whether the reduced level of viral mRNA in OTF963 cells was due to a reduced level of transcriptional initiation, rather than differences in mRNA stability, nuclear run-on assays were per-

formed with nuclei isolated from OTF963 and differentiated OTF963 cells at various times after infection. Results from the nuclear run-on assay showed that very little viral transcription occurred from any early viral transcription unit in undifferentiated cells 16 to 40 h postinfection (Fig. 5). At 48 h postinfection, the transcription rate for all genes increased, and by 64 to 72 h postinfection, viral transcription rates in OTF963 cells were similar to those in differentiated OTF963 cells 16 to 24 h postinfection. The fact that all viral genes were activated at a similar time suggests that one of the limiting factors for viral transcriptional activation in OTF963 cells is the expression of the E1a gene, which in turn transactivates the other viral early genes.

Transcriptional initiation, as measured by nuclear run-on data, was quantitated by using densitometry of the appropriate autoradiographic exposures and normalized for α -tubulin transcription. The profile of viral transcriptional initiation

FIG. 5. Viral transcriptional initiation rate in OTF963 and differentiated OTF963 cells. Cells were infected at 20 iu per cell, and at the indicated times nuclei were isolated. Radiolabeled run-on products were made as described in Materials and Methods and hybridized to alkaline denatured plasmids containing the indicated adenovirus genes fixed to Zeta Probe membranes.

FIG. 6. Immunoprecipitation of E1a, E1b, and hexon proteins in OTF963 and differentiated OTF963 cells. Cells were infected at 20 iu per cell and at the indicated hours after infection (lanes 24, 48, and 72) were incubated in [35 S]methionine-containing medium for 2 h prior to isolation of proteins for immunoprecipitation. Proteins immunoprecipitated by M73 (E1a) (panel a), H14b (E1b) (panel b), or a polyclonal antiserum to hexon (panel c) were fractionated by SDS-polyacrylamide gel electrophoresis on a 9% polyacrylamide gel and viewed by fluorography.

followed very closely that seen at the level of mRNA abundance, which indicates that viral mRNA stability is not a major factor leading to the reduced levels of viral mRNA in OTF963 cells.

These results show that E2 and E3 are more actively expressed than other adenovirus genes in OTF963 cells at the level of mRNA transcription as well as mRNA abundance, but at both levels E2 and E3 transcripts were reduced in OTF963 cells compared with differentiated OTF963 cells. This suggests that if there is E1a-like activity in OTF963 cells, it could be activated E2F, which selectively activates E2 and possibly E3 (55) but not other adenovirus genes. However, this activity is restricted both quantitatively and in terms of the range of target promoters it activates compared with viral E1a.

Adenovirus protein synthesis parallels reduced mRNA levels in OTF963 cells. In order to determine if reduced levels of viral transcripts were reflected at the level of protein synthesis, early (E1a and E1b) and late (hexon) viral antigens were immunoprecipitated from infected OTF963 and differentiated OTF963 cells. In differentiated cells, viral protein synthesis reached a maximum by 24 h postinfection for all genes tested and remained at this level until 72 h. In undifferentiated OTF963 cells, however, there were very low levels of E1a, E1b, and hexon viral protein synthesis up to 48 h postinfection (Fig. 6). By 72 h postinfection, viral protein synthesis in OTF963 cells had increased; E1a and E1b protein levels exceeded those at 24 h after infection of differentiated OTF963 cells, but the amount of hexon protein produced in undifferentiated cells never reached the level in differentiated cells 24 to 48 h after infection. Quantitative comparison in OTF963 cells and their differentiated derivatives of the relative amounts of adenovirus mRNA transcription and abundance and protein synthesis showed that they varied in parallel.

An interesting observation was the coprecipitation of E1a products with a protein of an apparent mobility of 105 to 110 kDa, the level of which did not vary as a function of differentiation (Fig. 6). This protein may be p105, the retinoblastoma gene product (52), or p107 (9), both of which have been implicated as antioncogenes.

As shown in Fig. 6, the antibody H14b precipitated the

E1b 58-kDa protein and a protein with the mobility of 53 kDa, which we thought might be the proto-oncogene p53. This was confirmed by coprecipitation of a 58-kDa protein with the p53 antibody pAb 200-47 (data not shown). p53 has previously been shown to coprecipitate with the E1b 58-kDa protein in transformed cells or in vitro (6) but not during adenovirus infection.

Viral DNA replication is reduced and delayed in OTF963 cells, and little infectious progeny virus is produced. To determine and compare the efficiency of viral DNA replication, OTF963 and differentiated OTF963 cells were infected and at various times viral DNA was detected by in situ hybridization. Viral DNA replication in differentiated OTF963 cells began before 16 h and plateaued by about 24 h after infection (Fig. 7a). In undifferentiated cells, however, viral DNA replication began after 24 h postinfection and plateaued at 48 h at about the level reached at 16 h in differentiated cells. The production of infectious progeny was also defective and delayed in OTF963 cells, with a yield of only about 0.2 iu per cell at 72 h, compared with more than 20 iu per cell in differentiated cells (Fig. 7b). It was not possible to continue the experiment to see if virus progeny increased further in OTF963 cells, because cells infected for more than 72 h lost viability.

DISCUSSION

As expected, we found that E1a transcription was at a low level in OTF963 cells but was much more active in differentiated cells. These differences were reflected at the levels of E1a mRNA abundance and protein synthesis. However, unlike papovavirus early genes, the defect in adenovirus E1a transcription was partially overcome, so that after 48 h of infection of OTF963 cells the level of transcription was similar to that 16 h after infection of differentiated cells. Lack of papovavirus transcription in F9 cells is due, at least in part, to repression of the early enhancer, and homology between the A domain of the polyomavirus enhancer and the E1a enhancer elements I and II (15) suggests that these elements may be involved in repression of adenovirus early gene expression in F9 cells. We have found positive and negative changes in proteins that bind to E1a enhancer

FIG. 7. (A) Viral DNA replication. OTF963 and differentiated OTF963 cells were infected at 20 iu per cell, and at the indicated times postinfection viral DNA was assayed by in situ hybridization and quantitated by using densitometry. (B) Production of mature infective virus. OTF963 and differentiated OTF963 cells were infected at a multiplicity of 20 iu per cell. At the indicated times postinfection, virus was harvested and titrated on HeLa cells and infectious viral production was calculated as infectious units per cell. ■, Differentiated OTF963 cells; □, undifferentiated OTF963 cells.

elements I and II when OTF963 cells differentiate (C. C. Nelson and A. J. D. Bellett, unpublished data). E1a-enhancer-linked genes are inefficiently transcribed in F9 cells following transfection, whereas efficient transcription occurs in differentiated F9 cells (22, 23, 29).

We expected from the hypothesis of an E1a-like factor in F9 cells (18) that in spite of the low level of expression of E1a, expression of other adenovirus genes would be normal. Our results, however, showed reduced transcriptional initiation of all adenovirus genes tested in OTF963 cells, as reflected by the levels of mRNA abundance, protein synthesis, viral DNA replication, and production of mature infectious viral progeny. Our results are consistent with an earlier comprehensive comparative study of adenovirus infection in the embryonal carcinoma cell lines F9-41, PYS, and PCC4 (7). In F9-41 cells, adenovirus early genes were repressed, DNA replication was delayed, late genes were severely repressed, and late proteins were not synthesized, compared with the undifferentiated pluripotent EC cell line PCC4 and the differentiated EC-derived cell line PYS. In an apparently conflicting report, E2 transcription, as measured by the nuclear run-on method, was found to be similar in F9 cells in comparison to differentiated F9 cells (54); however, if expression were normalized for differences in actin expression apparent in the data, it seems that the results would be consistent with those reported here. We also found that

adenovirus early gene expression was dependent on viral E1a in both OTF963 EC cells and their retinoic acid-differentiated counterparts.

If OTF963 cells do contain an E1a-like factor, then it is much less efficient at transactivating E2a than viral E1a, as judged by comparing E2 mRNA levels in wild-type- and *d1312*-infected cells, and has a much narrower promoter range than viral E1a. Some authors have suggested that the F9 E1a-like factor is an activated form of E2F, which is involved in transactivation of E2 rather than other adenovirus genes, with one or two possible exceptions (20, 55). A small amount of E2F activity in OTF963 cells may be responsible for the observation that E2 and E3 were consistently expressed at slightly higher levels than E1a, E1b, and E4, although E2 expression was still about 15-fold less in OTF963 EC cells compared with differentiated cells. Others have reported that F9 cells contain high levels of *c-myc* which are rapidly lost upon differentiation (8). OTF963 cells do not contain an increased level of *c-myc* (28, 41; C. C. Nelson, unpublished data), a cellular gene which is transactivated by E2F (16). *c-myc* has been reported to transactivate the adenovirus genes E2a and E4 (33). E4 transcription is the most defective of the adenovirus early genes in OTF963 cells. Neither E2F nor *c-myc* has been reported to repress papovavirus enhancers; moreover, these enhancers are repressed in undifferentiated OTF963 cells, which do not have elevated *c-myc* (28, 41, 42). Thus, there appear to be limits to the generality and explanatory power of the hypothesis that F9 cells contain a cellular E1a-like factor lost upon differentiation. Although some F9 lines may have high levels of E2F and/or *c-myc*, this is not true for all F9 lines nor are these cellular genes qualitatively or quantitatively equivalent to viral E1a.

A comparison of adenovirus expression in OTF963 and differentiated OTF963 cells over time has consistently shown an initial lag phase of expression between 0 to 48 h after infection of undifferentiated OTF963 cells, followed by a phase of increased transcription, which resulted at 48 to 72 h in levels of early gene expression similar to those 16 h after infection of differentiated OTF963 cells. An investigation of the transition in OTF963 cells from minimal to activated transcription of adenovirus genes was undertaken (C. C. Nelson, A. W. Braithwaite, M. J. Silvestro, and A. J. D. Bellett, submitted for publication). The results suggest that small amounts of viral E1a gene products made early in infection of OTF963 cells mediate differentiation-specific and other cellular changes which result in subsequent activation of adenovirus E1a transcription in an autocatalytic feedback loop.

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LITERATURE CITED

1. Babiss, L. E. 1989. The cellular transcription factor E2f requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression. *J. Virol.* **63**:2709-2717.
2. Bagchi, S., P. Raychaudhuri, and J. R. Nevins. 1989. Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system. *Proc. Natl. Acad. Sci. USA* **86**:4352-4356.

3. Bellett, A. J. D., P. Li, E. T. David, E. J. Mackay, A. W. Braithwaite, and J. R. Cutt. 1985. Control functions of adenovirus transformation region E1A gene products in rat and human cells. *Mol. Cell. Biol.* 5:1933-1939.
4. Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp. 1979. Pre-early Ad5 gene product regulates synthesis of early viral mRNAs. *Cell* 17:935-944.
5. Borrelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature (London)* 312:608-612.
6. Braithwaite, A. W., and J. R. Jenkins. 1989. Ability of p53 and the adenovirus E1b 58-kilodalton protein to form a complex is determined by p53. *J. Virol.* 63:1792-1799.
7. Cheng, C., and J. Praszkiar. 1982. Regulation of type 5 adenovirus replication in murine teratocarcinoma cell lines. *Virology* 123:45-59.
8. Dean, M., R. A. Levine, and J. Campisi. 1986. *c-myc* regulation during retinoic acid-induced differentiation of F9 cells is post-transcriptional and associated with growth arrest. *Mol. Cell. Biol.* 6:518-524.
9. Dyson, N., K. Buchkovich, P. Whyte, and E. Harlow. 1989. The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. *Cell* 58:249-255.
10. Feuer, G., M. Taketo, R. Hanecak, and H. Fan. 1989. Two blocks in Moloney murine leukemia virus expression in undifferentiated F9 embryonal carcinoma cells as determined by transient expression assays. *J. Virol.* 63:2317-2324.
11. Fujimura, F. K., P. L. Deininger, T. Friedman, and E. Linney. 1981. Mutation near the polyoma DNA replication origin permits productive infection in F9 embryonal carcinoma cells. *Cell* 23:809-814.
12. Gautsch, J. W., and M. C. Wilson. 1983. Delayed *de novo* methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression. *Nature (London)* 301:32-37.
13. Gorman, C. M., P. W. J. Rigby, and D. P. Lane. 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. *Cell* 42:519-526.
14. Grover, A., and E. D. Adamson. 1986. Evidence for the existence of an early common biochemical pathway in the differentiation of F9 cells into visceral or parietal endoderm: modulation by cyclic AMP. *Dev. Biol.* 114:492-503.
15. Hearing, P., and T. Shenk. 1986. The adenovirus type 5 E1A enhancer contains two functionally distinct domains: one is specific for E1A and the other modulates all early units in cis. *Cell* 45:229-236.
16. Hiebert, S. W., M. Lipp, and J. R. Nevins. 1989. E1A-dependent trans-activation of the human myc promoter is mediated by the E2F factor. *Proc. Natl. Acad. Sci. USA* 86:3594-3598.
17. Hogan, B. L. M., D. Barlow, and R. Tilly. 1983. F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm. *Cancer Surv.* 2:115-140.
18. Imperiale, M. J., H. Kao, L. T. Feldman, J. R. Nevins, and S. Strickland. 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol. Cell. Biol.* 4:867-874.
19. Jones, N., and T. Shenk. 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA* 76:3665-3669.
20. Jones, N. C., P. W. J. Rigby, and E. B. Ziff. 1988. Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. *Genes Dev.* 2:267-281.
21. Kao, H., and J. R. Nevins. 1983. Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. *Mol. Cell. Biol.* 3:2058-2065.
22. Kellermann, O., M. Buc-Caron, and J. Gaillard. 1987. Immortalization of precursors of endodermal, neuroectodermal, and mesodermal lineages, following the introduction of the simian virus (SV40) early region into F9 cells. *Differentiation* 35:197-205.
23. Kellermann, O., and F. Kelly. 1986. Immortalization of early embryonic cell derivatives after the transfer of the early region of simian virus 40 into F9 teratocarcinoma cells. *Differentiation* 32:74-81.
24. La Thanque, N. B., and P. W. J. Rigby. 1988. The regulation of SV40 early gene expression in embryonal carcinoma stem cells; faithful transcriptional regulation *in vitro*. *Nucleic Acids Res.* 16:11417-11430.
25. Linder, S., U. Krondahl, R. Sennerstam, and N. R. Ringertz. 1981. Retinoic acid-induced differentiation of F9 embryonal carcinoma cells. *Exp. Cell Res.* 132:453-460.
26. Linial, M., N. Gunderson, and M. Groudine. 1985. Enhanced transcription of *c-myc* in bursal lymphoma cells requires continuous protein synthesis. *Science* 230:1126-1132.
27. Linney, E., B. Davis, J. Overhauser, E. Chao, and H. Fan. 1984. Non-function of a Moloney murine leukemia virus regulatory sequence in F9 embryonal carcinoma cells. *Nature (London)* 308:470-472.
28. Lockett, T. J., and M. J. Sleight. 1987. Oncogene expression in differentiating F9 mouse embryonal carcinoma cells. *Exp. Cell Res.* 173:370-378.
29. Montano, X., and D. P. Lane. 1987. The E1a gene induces differentiation of F9 teratocarcinoma cells. *Mol. Cell. Biol.* 7:1782-1790.
30. Nermut, M. V. 1987. Adenoviridae. In M. V. Nermut and A. C. Steven (ed.), *Animal virus structure*. Elsevier Science Publishers B.V., Amsterdam.
31. Nevins, J. R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* 26:213-220.
32. Niwa, O., Y. Yokota, H. Ishida, and T. Sugahara. 1983. Independent mechanisms involved in suppression of the Moloney leukemia virus genome during differentiation of murine teratocarcinoma cells. *Cell* 32:1105-1113.
33. Onclercq, R., P. Gilardi, A. Lavenu, and C. Cremisi. 1988. *c-myc* products *trans*-activate the adenovirus E4 promoter in EC stem cells by using the same target sequence as E1a products. *J. Virol.* 62:4533-4537.
34. Paeratakul, U., P. R. de Stasio, and M. W. Taylor. 1988. A fast and sensitive method for detecting specific viral RNA in mammalian cells. *J. Virol.* 62:1132-1135.
35. Reichel, R., I. Kovesdi, and J. R. Nevins. 1987. Developmental control of a promoter-specific factor that is also regulated by the E1A gene product. *Cell* 48:501-506.
36. Reichel, R., S. D. Neill, I. Kovesdi, C. Simon, P. Raychaudhuri, and J. R. Nevins. 1989. The adenovirus E4 gene, in addition to the E1A gene, is important for *trans*-activation of E2 transcription and for E2F activation. *J. Virol.* 63:3643-3650.
37. Rosenstrauss, M. J., and A. J. Levine. 1979. Alterations in the developmental potential of embryonal carcinoma cells in mixed aggregates of nullipotent and pluripotent cells. *Cell* 17:337-346.
38. Rosenstrauss, M. J., C. L. Sundell, and R. M. Liskay. 1982. Cell-cycle characteristics of undifferentiated and differentiating embryonal carcinoma cells. *Dev. Biol.* 89:516-520.
39. Ross, S. R., A. J. Levine, R. S. Galos, J. Williams, and T. Shenk. 1980. Early viral proteins in HeLa cells infected with adenovirus type 5 host range mutants. *Virology* 103:475-492.
40. Sejersen, T., J. Sumegi, and N. R. Ringertz. 1985. Expression of cellular oncogenes in teratoma-derived cell lines. *Exp. Cell Res.* 160:19-30.
41. Sleight, M. J. 1987. Differential regulation of viral and cellular enhancers in F9 embryonal carcinoma cells. *Nucleic Acids Res.* 15:9379-9395.
42. Sleight, M. J., and T. J. Lockett. 1985. SV40 enhancer activation during retinoic acid-induced differentiation of F9 embryonal carcinoma cells. *EMBO J.* 4:3831-3837.
43. Sleight, M. J., T. J. Lockett, J. Kelly, and D. Lewy. 1987. Competition studies with repressors and activators of viral enhancer function in F9 mouse embryonal carcinoma cells. *Nucleic Acids Res.* 15:4307-4323.
44. Strickland, S., and V. Mahdavi. 1978. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* 15:393-403.
45. Strickland, S., and M. J. Sawey. 1980. Studies on the effects of

- retinoids on the differentiation of teratocarcinoma cells *in vitro* and *in vivo*. *Dev. Biol.* **78**:76–85.
46. Strickland, S., K. K. Smith, and K. R. Marotti. 1980. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* **21**:347–355.
47. Swartzendruber, D. E., T. D. Friedrich, and J. M. Lehman. 1977. Resistance of teratocarcinoma stem cells to infection with simian virus 40: early events. *J. Cell. Physiol.* **93**:25–30.
48. Swartzendruber, D. E., and J. M. Lehman. 1975. Neoplastic differentiation: interaction of simian virus 40 and polyoma virus with murine teratocarcinoma cells. *J. Cell. Physiol.* **85**:179–188.
49. Teich, N. M., R. A. Weiss, G. R. Martin, and D. R. Lowy. 1977. Virus infection of murine teratocarcinoma stem cells lines. *Cell* **12**:973–982.
50. Trevor, K., and J. M. Lehman. 1982. The interaction of polyoma virus with F9 embryonal carcinoma cells and chemically induced differentiated progeny: fate of the viral DNA and expression of viral antigens. *J. Cell. Physiol.* **2**(Suppl.):69–83.
51. Velcich, A., and E. Ziff. 1985. Adenovirus E1a proteins repress transcription from the SV40 early promoter. *Cell* **40**:705–716.
52. Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature (London)* **334**:124–129.
53. Yee, A. S., R. Reichel, I. Kovesdi, and J. R. Nevins. 1987. Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multicomponent complex. *EMBO J.* **6**:2061–2068.
54. Young, K. S., R. Weigel, S. Hiebert, and J. R. Nevins. 1989. Adenovirus E1A-mediated negative control of genes activated during F9 differentiation. *Mol. Cell. Biol.* **9**:3109–3113.
55. Zajackowski, D. A., P. Jalinot, and C. Kedinger. 1988. E1a-mediated stimulation of the adenovirus E1II promoter involves an enhancer element within the nearby E1IIa promoter. *J. Virol.* **62**:1762–1767.