Adenovirus infection of differentiated F9 cells results in a global shut-off of differentiation-induced gene expression

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ABSTRACT

Previous experiments have demonstrated a link between transcriptional regulatory mechanisms acting during F9 cell differentiation and transcription control by the adenovirus E1A gene. We have isolated a number of differentiation-specific genes by cDNA cloning to determine if E1A exerts a coordinated control over differentiation specific gene expression. The mRNAs encoded by these cDNAs were undetectable or only barely detectable in undifferentiated cells but then rose in concentration upon differentiation. Analysis of transcription rates in isolated nuclei revealed that all but one of the genes was transcriptionally regulated during differentiation. Interestingly, α2-type IV collagen expression was activated by a post-transcriptional mechanism since the gene was transcribed in both undifferentiated and differentiated cells whereas the cytoplasmic mRNA was undetectable in undifferentiated cells but rose in abundance in parallel with other regulated transcripts. Adenovirus infection of differentiated F9 cells reduced the cytoplasmic mRNA levels of each of the differentiation specific genes to near that found in the undifferentiated cell. Of those genes that were transcriptionally activated by differentiation, adenovirus infection specifically inhibited transcription. In contrast, although the α2 collagen mRNA levels were reduced by adenovirus infection similar to the other mRNAs, the control was post-transcriptional since transcription of the gene was unaffected. Thus, the mechanism for loss of gene expression mediated by E1A reflects the mechanism by which the gene was activated during differentiation. Based on these results we suggest that E1A controls the expression of the F9 cell phenotype by targeting a regulatory activity acting early in the differentiation program.

INTRODUCTION

The adenovirus E1A oncogene in conjunction with the *ras* gene or the adenovirus E1B gene is capable of transforming primary cells to an oncogenic state (1−3). In so doing, the E1A gene appears to provide an immortalizing function that enables cells to continually proliferate. The E1A gene is in fact a complex array of products that possess a variety of functions, including transcription activation and transcription repression (4−6). Early in a lytic viral infection and in transformed cells, 13S and 12S messenger RNAs are produced encoding proteins of 289 and 243 amino acids, respectively (7−9). The 289 aa product is a strong *trans*-activator of transcription of early adenovirus genes (10−12) and the 46 aa unique to this protein appear to be critical for this function (13−15). The 243 aa product can also *trans*-activate transcription (16−21) and recent experiments have suggested that a part of this activity is due to the ability of E1A to dissociate a transcription factor from a complex with a cellular protein (22). Finally, a variety of experiments have demonstrated that the transcriptional repressing activity of E1A is also dependent upon sequence within the 243 aa product of the 12S E1A mRNA. The negative regulation of transcription by the E1A gene product targets various viral enhancer elements including the SV40 enhancer and the polyomavirus enhancer (23−25). Other studies have shown that cellular genes can be subject to repression by the E1A gene product and this often involves cellular genes that are expressed in a cell-specific manner such as the insulin gene (26), the immunoglobulin heavy chain (27) and light chain loci (28). However, despite intensive efforts to define the basis for E1A-dependent repression, the actual mechanism is not understood including whether the repression of a given gene is directly mediated by E1A or whether E1A alters the action of an upstream regulatory gene that ultimately leads to the loss of expression of the target.

Previous experiments have suggested intriguing parallels between the control of gene expression during F9 teratocarcinoma cell differentiation and transcription control mediated by the E1A oncogene. Teratocarcinoma cells are malignant stem cells arising from germ tissue that are capable of differentiating *in vitro* into a variety of cell types (29, 30). The capacity to achieve *in vitro* differentiation following treatment with retinoic acid and cAMP (31, 32) led to the use of the F9 cell system as a convenient *in vitro* model for early events of mouse development, particularly the regulation of gene expression during this process. Other experiments have demonstrated a regulatory activity in undifferentiated F9 cells similar in nature to the adenovirus E1A gene product in the sense that F9 cells could complement an E1A deletion mutant with respect to early transcription activation (33). This complementing activity is developmentally regulated since upon differentiation, the activity disappears. Additional evidence that this activity functions similarly to E1A comes from the observation that the E2F transcription factor, a target for E1A

control in a viral infection (34, 35) is also regulated by F9 cell differentiation (36).

The differentiation of F9 cells also affects the control of other viral genes. For instance, the early transcription units of polyoma virus and SV40 are inactive in the undifferentiated cell, but are activated upon cell differentiation (37–41). This control is transcriptional, appears to be a function of interactions at the viral enhancers (42–45), and in part is due to negative control imposed by the undifferentiated F9 cell (46, 47). These observations thus suggest certain common aspects of transcription control, both positive and negative, mediated by E1A and by F9 cell activities and have prompted the idea that the control of gene expression by an activity functioning similarly to the viral E1A may be important in the differentiation process (48). This is also supported by studies demonstrating that E1A can block differentiation of certain cell lines (49, 50). Thus, the study of the manner by which E1A affects gene control and differentiation in F9 cells may be informative with respect to normal differentiation processes. The work we describe in this paper addresses the more general question of the role of an E1A-like activity in the overall control of gene expression during differentiation through an analysis of a group of genes controlled by the differentiation process.

METHODS

Cells and viruses

The F9 teratocarcinoma cell line, obtained from E. Linney, was maintained in DME containing 10% fetal calf serum (Gibco) as previously described (48). Cultures were induced to differentiate by the addition of retinoic acid (Sigma) to 10^{-7}M and dibutyryl cAMP (Sigma) to 10^{-3}M. Stocks of adenovirus-5 and the E1A deletion mutant d1312, were prepared as previously described (51). Virus stocks were titered on 293 cells by fluorescent focus assay (19). For virus infection, cells were infected at a multiplicity of 50 ffu per cell.

RNA isolation and analysis

RNA was isolated from F9 cells or differentiated F9 cells as described (48). Northern analysis of RNA was performed as described before (48).

cDNA cloning

Double-stranded cDNA was constructed from polyadenylated RNA isolated from differentiated F9 cells using a cDNA synthesis kit (Amersham). The cDNA was ligated to EcoRI linkers (BRL) and excess linkers were digested with EcoRI and removed by chromatography over Sephacryl 300 S (Sigma). The cDNA was ligated into λZAP (Stratagene) and packaged using Gigapak Gold extract. Phage were titered and screened on BB4 hosts as described by Stratagene. Lambda phage recombinants with inserts of genes specific to the differentiated state were identified by differential screening (See Fig. 1). Identical plaque lifts were probed with cDNA probes prepared from undifferentiated and differentiated F9 cell mRNA. Phage which hybridized to the differentiated F9 cDNA probe but not the undifferentiated probe

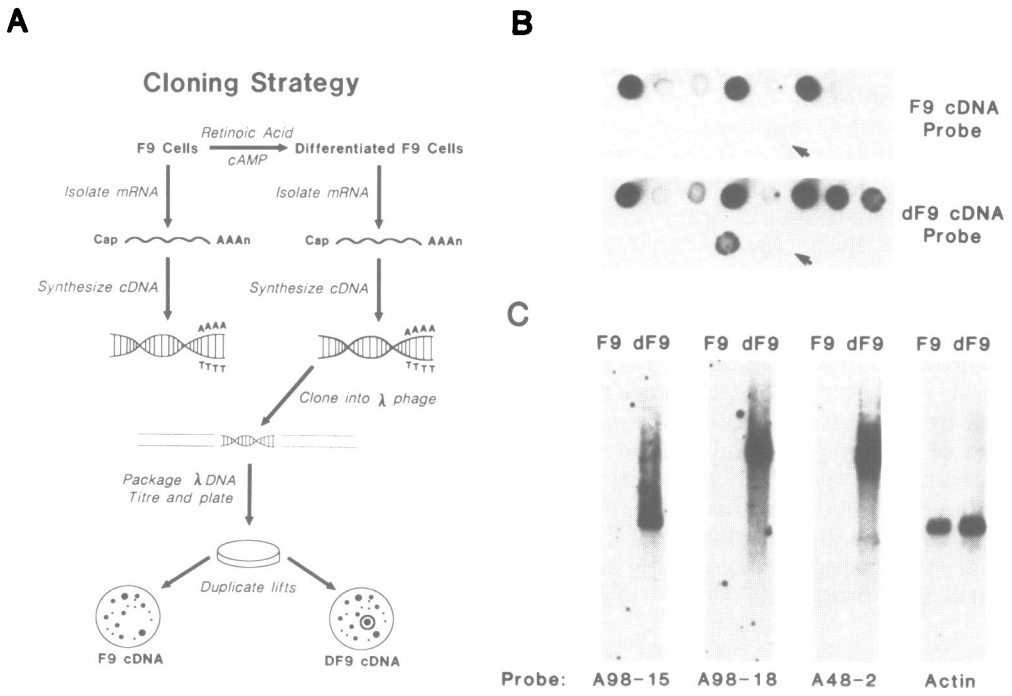

Figure 1. Scheme for the isolation of differentiation-specific F9 cell cDNAs. A. Schematic for the selection of cDNAs. B. Example of secondary screening revealing differentially hybridizing clones; note that there are three clones which clearly hybridize to the dF9 probe but not the F9 cDNA probe. Arrow demonstrates λZap control. C. Northern analysis of RNA from undifferentiated F9 cells and differentiated F9 cells probed with several cDNAs as well as actin.

were picked and subjected to a second screen. Approximately 30% of these phage hybridized specifically to a differentiated F9 cell cDNA probe on the secondary screen. Inserts from λZAP recombinant phage were recovered as Blue Script plasmids as described by Stratagene. These plasmids were then used as probes of Northern blots of mRNA isolated from undifferentiated or differentiated F9 cells. The inserts were sequenced as described by Sanger (52).

Isolated nuclei transcription assays

The procedures for preparation of nuclei and assay of transcription rates have been described (53).

RESULTS

Differentiation-specific cDNAs

Several genes have previously been described whose expression is induced upon differentiation of F9 cells, including laminin B1 (54), tissue plasminogen activator (tPA) (55), type IV collagen (54, 56) and genes encoding the major histocompatibility complex (MHC) proteins (54). Our previous studies have demonstrated that the expression of two of these genes, tPA and collagen, is repressed by adenovirus infection, dependent on E1A function (48). In an attempt to determine the extent of the negative control by E1A, we have constructed a cDNA library from differentiated

Table I. Characteristics of differentiation-specific cDNA clones

λZap recombinant	Plasmid	Approximate insert size	mRNA size (Kb)	Gene identified by sequence homology
A40-21	A48-2	2.3	8.4	α1 type IV collagen
A40-28	A48-10	2.5*	8.4	α1 type IV collagen
A79-33	A83-3	2.3	8.4	α1 type IV collagen
A72-6	A83-5	1.5	8.4	α2 type IV collagen
A72-10	A98-18	2.8*	8.4	α2 type IV collagen
A72-23	A83-9	1.6	7.3	laminin B1
A82-4	A98-21	1.7	7.3	laminin B1
A72-1	A98-1	1.6°	2.5	protein disulfide isomers
A82-5	A98-24	1.6°	ND	protein disulfide isomers
A72-8	A98-15	0.9	2.1	SPARC
A64-1	A70-1	2.5°	12.0	laminin A
A64-47	A70-3	1.5°,*	2.1	unknown

° Contains an internal EcoRI site.
* At least one EcoRI site is missing and/or cDNA may be involved in re-arrangement.
ND = Not done.

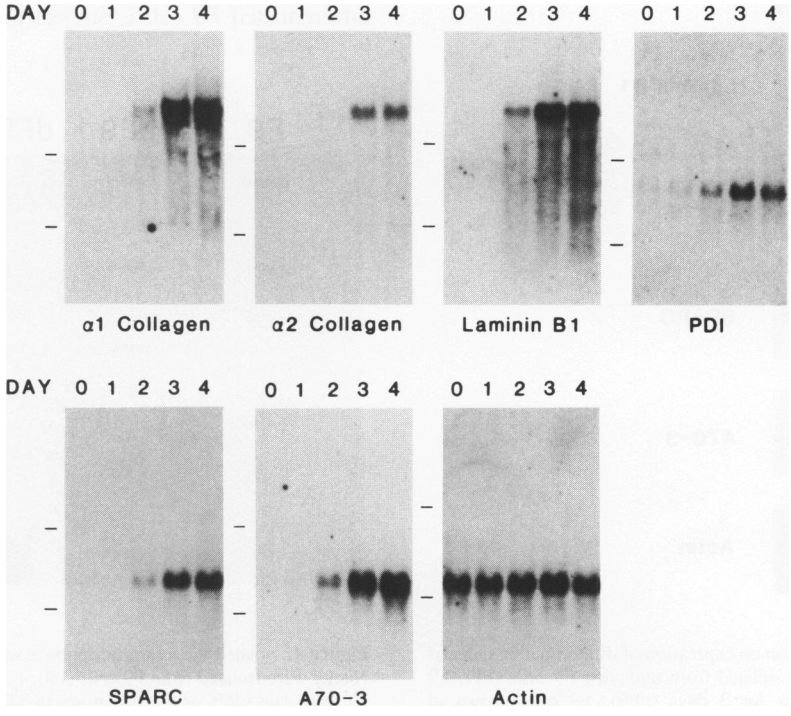

Figure 2. Kinetics of activation of differentiation-specific F9 mRNAs. Total cytoplasmic RNA was isolated from F9 cells untreated (Day 0) or treated with retinoic acid and dibutyryl cAMP (differentiation media) for 1, 2, 3, or 4 days. Identical Northern blots were probed with each of the dF9-specific cDNAs as well as actin as a control. Hash marks to left of each blot indicate the location of 28S and 18S ribosomal RNAs.

F9 cell RNA and isolated cDNA clones of mRNAs specifically induced by differentiation following the strategy depicted in Figure 1. After screening approximately 15,000 recombinant phage, twelve were isolated based on specific hybridization to cDNA from differentiated F9 cell RNA. Each of these clones were then used as probes on Northern blots as depicted in Figure 1. Inserts from these clones were then sequenced and the sequence compared to GenBank sequences to identify each gene. Of the twelve clones, seven different genes were represented (Table I). Six of these were clones of previously identified genes including α1 type IV collagen, α2 type IV collagen, laminin B1, laminin A, protein disulfide isomerase (PDI), and SPARC (secreted, acidic, cysteine-rich glycoprotein). One cDNA, A70−3, contained sequences which have not previously been described. This plasmid hybridizes to a 2.1 kb mRNA.

An analysis of the kinetics of activation of each of the genes following addition of retinoic acid and cAMP is shown in Figure 2. Cytoplasmic RNA was isolated from untreated F9 cells or cells treated for 1, 2, 3, and 4 days with retinoic acid and cAMP and then analyzed by Northern blotting. Identical blots were probed with six of the cDNA clones or an actin cDNA clone as a control. The laminin A clone is not represented since we have not been able to obtain reproducible Northern analyses of this mRNA in virus infection experiments without extensive degradation, presumably due to the large size of the mRNA. Each of the genes represented by the cDNAs was activated with approximately the same kinetics during the differentiation process, with a peak level reached at 72 hours. The use of γ-actin as a probe confirmed that approximately equal amounts of RNA were applied to each lane. These data demonstrate that the expression of each of these clones was characteristic of the differentiated state of the cells and thus the expression of this group of genes could serve as a marker for the phenotypic changes associated with the differentiation process.

Effects of adenovirus infection on expression of differentiation-specific genes

Using these cDNAs as probes, we have examined the effect of adenovirus infection on the regulation of cellular gene expression during differentiation of F9 cells. Cells were treated for two days with retinoic acid and cAMP, then infected with wild-type Ad5 or d1312, a deletion mutant deficient in E1A expression. RNA was isolated 24 hours later and analyzed by a Northern. As shown in Figure 3, Ad5 infection resulted in a significant reduction in the level of each of the differentiation-specific transcripts, with the exception of PDI which was reduced but not as dramatically as the others. Since infection with d1312 had little or no effect on differentiation-specific gene expression, we conclude that E1A was necessary for the repression. Combining this result with our previous analyses that demonstrated a loss of tPA and α1 collagen expression upon adenovirus infection (48), we conclude that the entire group of genes whose expression is characteristic of the differentiated F9 cell is subject to E1A-dependent repression.

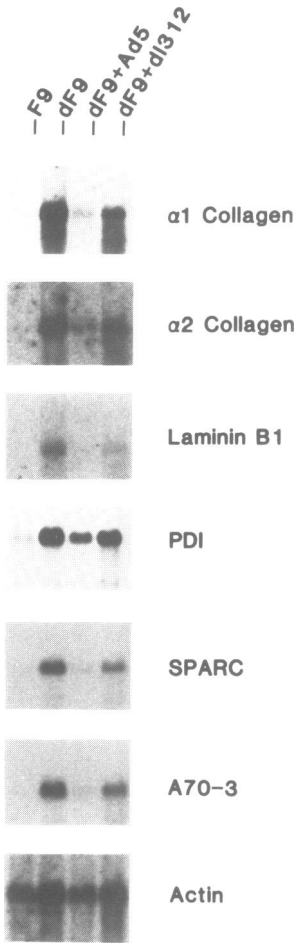

Figure 3. Effect of adenovirus infection on expression of differentiation induced RNAs. Total cytoplasmic RNA was isolated from untreated F9 cells (F9), F9 cells grown in differentiation media for 3 days (dF9), F9 cells grown in differentiation media for 2 days and then infected with wild type adenovirus 5 (dF9+Ad5) or d1312 (dF9+d1312). After infection, cells were grown in differentiation media for an additional 24 hours prior to RNA isolation. Identical Northern blots were probed with each differentiation-specific cDNA or actin as a control.

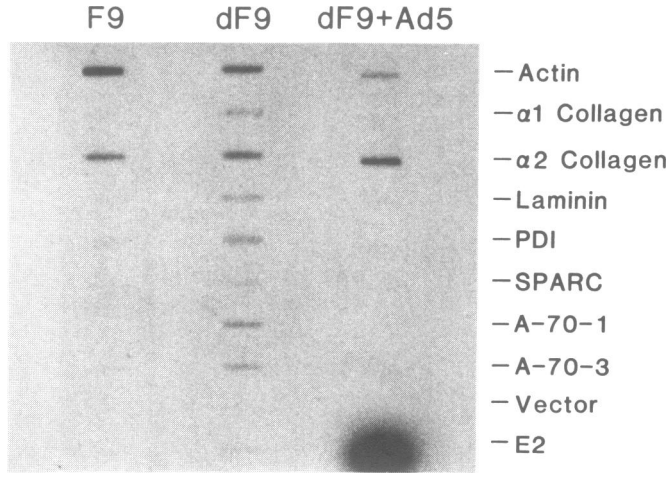

Figure 4. Isolated nuclei transcription assays for differentiation-specific genes. Nuclei were isolated from F9 cells (F9), F9 cells grown in differentiation media for three days (dF9) or F9 cells grown in differentiation media for 2 days, infected with wild type adenovirus 5 and incubated an additional 24 hours with differentiation media prior to analysis (dF9+Ad5). Nuclei were labeled with α^{32}P-UTP for 15 minutes at 30°C. RNA was extracted and used to probe nitrocellulose blots bearing 5 μg of each cDNA. Actin, vector alone and E2 (a plasmid containing coding sequence for adenovirus E2 gene) were used as controls.

Differentiation-specific transcripts are controlled by transcriptional and post-transcriptional mechanisms

Nuclear run-on assays were used to examine the transcriptional rates of each of these genes. Nuclei from F9 cells, differentiated F9 cells, or differentiated cells infected with Ad5 were labeled for 15 minutes with α-^{32}P UTP. The labeled RNA was extracted and assayed by hybridization to DNA slot blots containing each of the cDNAs. With the exception of the α2 type IV collagen gene, each of the differentiation-specific genes appeared to be transcriptionally regulated as a function of differentiation (Fig. 4). Transcription of each gene was undetectable or very low in the undifferentiated F9 cells but increased markedly upon differentiation, although the signal obtained with the SPARC probe was only slightly above background. We also included the laminin A clone in this assay since a transcription measurement is not affected by the lability of the mRNA. Clearly, transcription of this locus behaved similarly to the others. In contrast to the transcription of these genes, actin was actively transcribed before and after differentiation. Surprisingly, the α2 type IV collagen gene was actively transcribed in undifferentiated F9 cells even though the mRNA could not be detected (see Fig. 2). Furthermore, there was no increase in transcription upon differentiation, conditions that resulted in a large increase in α2 collagen mRNA levels. We thus conclude that the α2 type IV collagen gene, unlike each of the others, is regulated by a post-transcriptional mechanism during differentiation.

For each of the genes subject to transcriptional regulation upon differentiation, adenovirus infection resulted in a repression of this transcription. That is, the transcription rate in virus infected cells dropped to near the level found in the undifferentiated cells. In this experiment, actin transcription was somewhat reduced but this was only two-fold and thus much less than that of other genes. In contrast, to the other differentiation-specific genes, transcription of the α2 collagen gene, which remained constant during differentiation, was unaffected by adenovirus infection despite the fact that the α2 collagen mRNA level was markedly reduced. We thus conclude that adenovirus infection results in a repression of the expression of each of the differentiation-specific genes and does so via the same level of control through which they are normally activated.

DISCUSSION

Since the development of the F9 cell line as a system to study cell differentiation *in vitro*, primarily as a result of the work of Strickland and colleagues that demonstrated the ability to induce differentiation with retinoic acid (31, 32), this system has proved invaluable in defining events that take place during the early stages of differentiation. Many studies have reported phenotypic characterizations of the differentiated state including the identification and isolation of genes whose expression is specific to the differentiated cell (54–58). However, the mechanisms controlling the differentiation process, including the relationship between cell proliferation and differentiation and the regulatory genes involved in this process, have remained obscure. This in part stems from the difficulty identifying the regulatory genes that are responsible for this control.

The results we present here clearly demonstrate that adenovirus infection, dependent on E1A function, represses the expression of an entire group of genes, the products of which are characteristic of the differentiated cell. The significance, we believe, lies not in the fact that E1A can shut off cellular gene expression, which has been demonstrated before, but rather in the fact that there is a coordinate loss of expression of a group of commonly regulated genes. Although it is possible that E1A directly represses transcription from each of the genes that are transcriptionally controlled, we view this possibility to be unlikely. Rather, we favor a mechanism whereby E1A alters the function of a regulatory gene or genes that acts at an earlier step in the differentiation process and whose product(s) may be important for the expression of the fully differentiated phenotype. We base this view on two observations. First, each gene that was activated by F9 cell differentiation was also repressed by adenovirus infection. Although there may be genes controlled by differentiation which do not respond to E1A, we have no evidence for such. Moreover, other cellular genes subject to E1A-mediated repression, such as insulin (26) and immunoglobulin heavy chain (27) and light chain (28), are also genes whose expression is characteristic of a differentiated cell. Although it is possible that targets of E1A action are found in each of these genes, we believe the more likely possibility, based on the apparent broad specificity of E1A negative control, is that E1A affects the expression of a gene or genes that is critical to initiate the differentiation program in F9 cells or other cell types.

Second, one of the F9 specific genes targeted by E1A, the α2 type IV collagen gene, is regulated by a post-transcriptional mechanism. Since there is no evidence to date to suggest that E1A can repress by a non-transcription mode, we infer that the control of α2 collagen is likely indirect and we thus suggest that all of the negative control might be indirect. We are therefore drawn to the hypothesis that E1A targets a regulatory mechanism acting early in the differentiation process and thereby represses expression of terminal differentiation markers. The significance of these results lies in the possibility that the study of E1A action may lead to the identification of a cellular regulatory activity involved in controlling the differentiation program.

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