E2F1 Messenger RNA Is Destabilized in Response to a Growth Inhibitor in Normal Human Keratinocytes but not in a Squamous Carcinoma Cell Line

Nicholas A. Saunders, Anthony J. Dicker, Susan J. Jones, and Alison L. Dahler

Abstract

Keratinocyte growth arrest is characterized by a reduction in the activity and expression of E2F1. Here, we examine the role posttranscriptional processing plays in the down-regulation of E2F1 during keratinocyte growth arrest. E2F1 mRNA levels were undetectable within 8 h of exposure to the protein kinase C activator, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Assays of transcript stability indicated that, in untreated keratinocytes, the half-life of E2F1 mRNA was 6.1 h and, in TPA-treated cells, it was 1.7 h. This destabilization was protein synthesis dependent. In contrast, a growth inhibitor-resistant carcinoma cell line, SCC25, had a very stable E2F1 half-life that was only moderately reduced following TPA treatment. These data demonstrate that the initiation of keratinocyte growth arrest is associated with a rapid destabilization of E2F1 mRNA. These data are consistent with the proposition that inactivation of the posttranscriptional processing of important growth regulatory genes (e.g., E2F1) may contribute to neoplasia.

Introduction

The transcription factor complex, E2F, refers to a heterodimeric complex consisting of an E2F family member (numbered 1–5) and a DP protein (numbered 1 or 2). This complex is an essential regulator of S-phase traverse in cycling cells (1). E2F1 was the first of the E2F family members to be cloned (2, 3), and there is now compelling functional evidence that E2F1 is a key regulator of the cell cycle as well as a potential oncogene (4, 5). For instance, forced expression of E2F1 can induce DNA synthesis as well as transform cells (4, 5). Although functional studies indicate that E2F1 is an important partner of Rb and S-phase traverse (6), there is also evidence, from E2F1 knockout mice (7, 8), that, in the absence of E2F1, other family members may substitute for its activity.

Given the causal association between E2F1 activity and the proliferation of established cell lines, we have examined the role of this transcription factor in keratinocyte proliferation. The results of these studies suggest that E2F1 may be a key player in keratinocyte proliferation/differentiation (9–11). Epithelial squamous differentiation is initiated by the irreversible growth arrest of proliferating keratinocytes followed by the expression of various differentiation-specific marker genes and activities (10). This pathway can be initiated, in vitro, by various agents (e.g., TPA, IFN-γ, senescence, or confluent monolayers of protein synthesis dependence of mRNA stability, protein synthesis was inhibited with 2.5 μg/ml cycloheximide (Sigma Chemical Co., St. Louis, MO).

Materials and Methods

Cell Culture and Treatments. HEKs were isolated from neonatal foreskins following circumcision and cultured as described (9). The keratinocyte-derived squamous carcinoma cell line, SCC25, was obtained from American Type Culture Collection (Rockville, MD) and cultured as described (9). Growth arrest of HEKs was induced by treatment with 50 ng/ml TPA (Fluka, Sydney, NSW, Australia), a protein kinase C activator, as described (12). The dose of actinomycin D required to inhibit transcription was determined by incubating HEKs or SCC25 cells with varying concentrations of actinomycin D (Sigma, Sydney, NSW, Australia) for 8 h. During the last 2 h, cells were coincubated with 5 μCi/ml (final concentration) [3H]uridine (ICN, Sydney, NSW, Australia; 43 Ci/mmol). At the end of the incubation, the cells were washed three times with PBS and then fixed in acid-alcohol (10% acetic acid-90% ethanol, v:v) at 4°C for 30 min, washed three times with PBS, and digested at 37°C for 30 min in 0.5 M NaOH. Digests were neutralized with an equivalent volume of 1 M HCl, and an aliquot was counted by liquid scintillation spectrometry. Uridine incorporation was normalized to protein. In studies of protein synthesis dependence of mRNA stability, protein synthesis was inhibited with 2.5 μg/ml cycloheximide (Sigma Chemical Co., St. Louis, MO).

RNA Isolation and Analysis. Isolation of RNA and analysis by RT-PCR (9) or Northern blotting (13) of E2F1 mRNA expression has been described. Quantitation of E2F1 mRNA expression levels was performed by PhosphorImager analysis of blots or, in some instances, by densitometric analysis of autoradiographs (9, 13). For E2F1 mRNA stability studies, the half-life of the...
mRNA was estimated by regression analysis of the log$_{10}$-transformed data. In the instance of the time course for E2F1 disappearance, the data were analyzed using the monoexponential equation $c = C_0 e^{-kt} + x$, where $c$ = observed E2F1 mRNA level, $C_0$ = initial E2F1 mRNA level, $k$ = degradation rate constant, $t$ = time, and $x$ = a linear component (12). Half-life was then estimated as 0.693/$k$. In all instances, E2F1 mRNA levels were normalized against those of GAPDH (for Northern analysis) or actin (RT-PCR analysis).

**Results**

Treatment of keratinocytes with 50 ng/ml TPA decreased E2F1 mRNA$_{ss}$ levels approximately 5-fold ($P < 0.05$; Fig. 1). This decrease in E2F1 mRNA$_{ss}$ levels was associated with a >95% decrease in DNA synthesis from 100 ± 24% in proliferating cells to 0.3 ± 0.1% in TPA-treated HEKs. In contrast, the treatment of the squamous carcinoma cell line SCC25 did not result in a decrease in E2F1 mRNA$_{ss}$ levels (Fig. 1) or DNA synthesis (100 ± 6% in untreated cells and 132 ± 8% in TPA-treated cells). Furthermore, treatment of HEKs with TPA also resulted in a fold induction of 10.9 ± 4.9 (n = 3) of the expression of the differentiation-specific marker gene, transglutaminase type I. TPA treatment did not induce differentiation in SCC25 cells, as measured by the expression of transglutaminase type I mRNA (data not shown). These data confirm our earlier findings that the treatment of HEKs with TPA induces a program of growth arrest and squamous differentiation (12). This pathway was not inducible in the SCC25 cells.

To establish whether posttranscriptional processing contributed to the decrease in E2F1 mRNA$_{ss}$ following TPA treatment, we examined a time course of E2F1 mRNA down-regulation in TPA-treated HEKs. Fig. 2 demonstrates that the disappearance of E2F1 mRNA is rapid. E2F1 mRNA levels are below 50% of controls following 4 h of TPA exposure and almost totally abolished by 8 h (Fig. 2). These data indicate that mechanisms affecting the regulation of E2F1 mRNA expression are initiated within the first 4 h of TPA treatment. Nonlinear regression analysis of the data in Fig. 2 estimates a $t_{1/2}$ of 2.1 ± 1.2 h ($n = 5$) for the disappearance of E2F1 mRNA$_{ss}$ levels in the presence of TPA alone.

Posttranscriptional processing of critical "growth genes" such as c-myc (14) and c-fos (15) have been reported previously. We, therefore, examined the possibility that alterations in the stability of E2F1 mRNA may contribute to the down-regulation of E2F1 mRNA$_{ss}$ levels observed following TPA treatment. Preliminary experiments indicated that a dose of 250 ng/ml actinomycin D for 8 h reduced transcription, estimated by $[^3]$H]uridine incorporation, to 1 ± 0.1% and 2.2 ± 0.22% of untreated HEKs and SCC25 cells, respectively.

Proliferating HEKs or SCC25 cells were left untreated or treated with 50 ng/ml TPA. Following a 4-h pretreatment (at which time E2F1 mRNA should be reduced in the TPA-treated cells; Fig. 2), 250 ng/ml (final concentration) actinomycin D was added, and the RNA was harvested at various times thereafter. The stability curves for E2F1 mRNA in HEKs and SCC25 cells are presented (Fig. 3). The $t_{1/2}$ for E2F1 in untreated HEKs (Fig. 3) was estimated to be 6.1 ± 2.0 h ($n = 12$), which was significantly greater than that observed in TPA-treated HEKs (1.7 ± 0.5 h, $n = 8$, $P < 0.05$, determined by F test). In contrast, the $t_{1/2}$ for E2F1 mRNA in untreated SCC25 cells was not determinable, and in the presence of TPA, it was approximately 7.2 ± 3.1 h ($n = 3$, Fig. 3). The long half-lives were not due to the inactivity of actinomycin D in the SCC25 cells because we had previously determined that 250 ng/ml actinomycin D reduced transcription in HEKs and SCC25 cells by 98%. Combined these data indicate that TPA treatment of HEKs leads to a destabilization of E2F1 mRNA. Furthermore, our data show that in the tumorigenic and growth inhibitor-resistant SCC25 cell line, the E2F1 mRNA $t_{1/2}$ is greater than in untreated HEKs and is resistant to TPA-mediated E2F1 mRNA destabilization.

Having established that E2F1 mRNA destabilization occurs during keratinocyte growth arrest/differentiation, we examined whether this required new protein synthesis. In this instance, untreated HEKs were treated with or without TPA, with or without cycloheximide, for 4 h and then exposed to actinomycin D for 3 h. Northern analysis revealed that cycloheximide had no effect on E2F1 mRNA$_{ss}$ levels in control HEKs but prevented the 5-fold down-regulation of E2F1 mRNA$_{ss}$ in TPA-treated HEKs (Fig. 4). These data suggest that the destabilization of E2F1 mRNA in response to TPA requires synthesis of a destabilizing protein or the loss of synthesis of a labile stabilizing protein. Alternatively, it may suggest that the destabilization is translation dependent. This latter possibility is less likely because no increase in E2F1 mRNA levels was noted in the control HEKs (Fig. 4, — TPA).

**Discussion**

Here, we have identified a novel regulatory pathway that is involved during the initial phase of keratinocyte differentiation. Previously, it...
had been reported that treatment of keratinocytes for 48 h with TPA results in a decrease in E2F1 mRNA and E2F activity. This study extends these findings to show that E2F1 mRNA levels are almost abolished 4–8 h following TPA treatment and that this correlates with an increase in the degradation rate of E2F1 mRNA. An estimate of the potential contribution of mRNA destabilization to the decrease in E2F1 mRNA levels observed during growth arrest is indicated by the similarity in half-life for HEKs treated with TPA alone (2.1 h) and that of the E2F1 mRNA degradation rate estimated in the presence of TPA and actinomycin D (1.7 h). Although E2F1 has been shown to be subject to significant transcriptional control during the cell cycle (16, 17), our data suggest that the destabilization of E2F1 mRNA may contribute entirely to the down-regulation of E2F1 mRNA observed during the initiation of keratinocyte growth arrest. Because an examination of E2F1 transcription rates was not undertaken in this study, it is not possible to quantify the relative contributions of these two processes to E2F1 mRNA down-regulation during keratinocyte differentiation. However, it is likely that E2F1 mRNA destabilization is involved because the increase in E2F1 mRNA degradation occurs rapidly and at a time when DNA synthesis is being switched off.

An important implication of this study is the possibility that the defect in posttranscriptional processing that was observed in the SCC25 cells may contribute to the growth inhibitor resistance of this carcinoma cell. This is supported by the relatively stable $t_{1/2}$ for E2F1 mRNA in untreated SCC25 cells and the attenuated ability to destabilize the E2F1 mRNA in response to TPA. Moreover, we have previously shown that the deliberate deregulation of E2F activity in normal keratinocytes leads to growth inhibitor resistance and a failure to down-regulate E2F1 mRNA (9). This latter observation would also indicate that the inability of SCC25 cells to down-regulate E2F1 mRNA is associated with growth inhibitor resistance (characteristic of tumor cells) rather than the immortalization process that occurs during the establishment of cell lines. Here, we demonstrate that the inability to down-regulate E2F1 mRNA in growth inhibitor-resistant cells may be due, in part, to a loss of posttranscriptional processing. Given that multiple genetic events are generally accepted to be required for the development of neoplasms, it is possible that one of these lesional events may target the posttranscriptional regulatory processing of the critical growth gene, E2F1. The inability to down-regulate E2F1 mRNA in response to growth inhibitors is also shared by several other keratinocyte-derived squamous carcinoma cells (10). This suggests that the findings of this study may reflect a more general phenomenon in epithelial tumorigenesis. The association of defective posttranscriptional processing and neoplasia has precedents. Previous studies have reported that defects in posttranscriptional processing of IL-3 (18) or fos (19) can contribute to their tumorigenicity.

Posttranscriptional processing is a mechanism by which the functions of many genes can be altered as a rapid response to external stimuli (20). A common means by which genes use posttranscriptional processing to modify cellular function is by altering the stability of the mRNA. Transcript stability involves both global regulatory mechanisms, such as mediation by the UUAUUUAUU sequence motif (ARE; Ref. 21) and cell- and gene-specific regulatory mechanisms (22). Transcripts for many genes (particularly cytokines and growth genes) contain single or multiple AREs within their 3' untranslated regions which, with few exceptions (e.g., interleukin 3; Ref. 23), are required but not sufficient for mRNA degradation. The AREs appear to act in concert with other gene-specific elements within the mRNA to target genes for stabilization or destabilization. This is demonstrated by the ability of TPA and IFN-γ to stabilize the ICAM-1

---

Fig. 3. E2F1 mRNA is destabilized in growth inhibitor-treated HEKs but not in SCC25 cells. Proliferating HEKs or SCC25 cells were pretreated with TPA (+ TPA) or without TPA (− TPA) for 4 h. Following pretreatment, actinomycin D (250 ng/ml, final concentration) was added (zero time on graph), and the RNA was harvested at various times thereafter. Total RNA (2 µg) was then reverse-transcribed in a 20-µl volume. A 1-µl aliquot was used to amplify either E2F1 or actin transcripts under linear conditions with respect to cycle number. The amplified product was then blotted and probed with [32P]cDNA probes for E2F1 or actin and subjected to PhosphorImager analysis. Data points, mean of three experiments; bars, SE (for HEKs).

Fig. 4. The destabilization of E2F1 mRNA is protein synthesis dependent. Proliferating HEKs were pretreated with TPA (+ TPA) or without TPA (− TPA) for 4 h. In some instances, the cells were also pretreated for 4 h with 2.5 µg/ml (final concentration) cycloheximide (3 h + chx), with or without TPA. Following pretreatment, the cells were treated with 250 ng/ml actinomycin D (final concentration), and RNA was harvested at either 0 or 3 h following the addition of actinomycin D. RT-PCR was analyzed as in Fig. 3. Column, mean percentage of the appropriate 0 h control from two experiments; bars, range.
mRNA in murine fibroblasts (22). Although both TPA and IFN-γ stabilize ICAM-1 mRNA in the same cell type, the mRNA sequence elements required for this are different (22). The E2F1 mRNA 3’ untranslated region contains two UUAUUUAUU motifs and one AUUUA motif (2, 3), which is consistent with its being a critical growth gene. However, because E2F1 mRNA is not down-regulated in human fibroblasts in response to growth inhibitors such as IFN-γ (24) or TPA, it may suggest that other cell type-specific factors are involved in the destabilization of E2F1 mRNA in differentiating keratinocytes.

The identification of the factors involved in the destabilization of E2F1 will provide insight into the regulation of keratinocyte differentiation and, possibly, skin tumor formation. To this end, our studies with cycloheximide provided some clues as to the mechanism by which TPA induced E2F1 mRNA destabilization. The apparent requirement for new protein synthesis suggested two possibilities. First, TPA may have induced the synthesis of a specific destabilizing protein that binds to a specific element within the E2F1 mRNA. Alternatively, TPA treatment may have inhibited the synthesis of a specific stabilizing factor. Preliminary RNA gel shift studies that are currently underway suggest the latter of these possibilities is the most likely.6

In conclusion, here, we demonstrate that a key cell cycle regulator, E2F1, is subject to posttranscriptional processing. In addition, this study demonstrates that keratinocyte growth arrest is associated with a rapid destabilization of E2F1 mRNA. Furthermore, we demonstrate that the inability of a carcinoma cell line to effectively destabilize E2F1 in response to a growth inhibitor was associated with its inability to undergo growth arrest. We speculate that the loss of posttranscriptional processing of E2F1 mRNA may contribute to the development of squamous carcinomas.

References

5 N. A. Saunders, unpublished data.
6 A. L. Dabler and N. A. Saunders, unpublished data.
E2F1 Messenger RNA Is Destabilized in Response to a Growth Inhibitor in Normal Human Keratinocytes but not in a Squamous Carcinoma Cell Line

Nicholas A. Saunders, Anthony J. Dicker, Susan J. Jones, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/58/8/1646

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.