The embryonal carcinoma stem cell Ela-like activity involves a differentiation-regulated transcription factor

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ABSTRACT

Murine F9 embryonal carcinoma (EC) stem cells have an Ela-like transcription activity that is undetectable in F9 cells differentiated to parietal endoderm-like cells (F9-PE). The Ela-inducible adenovirus E2A promoter has been used to further define this activity and we show that in vitro the transcription of this promoter in F9 EC and F9-PE cell extracts reflects the regulation in vivo. In EC cell extracts several trans-acting protein factors bind to E2A promoter sequences. A distal domain containing a CRE binds proteins present in F9 EC, F9-PE and Hela cell extracts. Sequences between -71 and -50 define a multiplicity of binding activities, termed DRTF1, all of which are down regulated as EC stem cells differentiate. DRTF2, a low abundance, regulated binding activity requires DNA sequences that overlap those required by DRTF1. The CRE and the DRTF1 binding site compete for transcription in vitro, indicating that in EC cell extracts the respective proteins function as positively acting, binding site dependent transcription factors. Comparison of DRTF1 with the previously defined HeLa cell factor E2F, induced during adenovirus infection, indicates that although both factors recognise the same region of the promoter there are clear differences between them. These data indicate that multiple factors are necessary for efficient transcription of the E2A promoter in F9 EC cell extracts and suggest that DRTF1 is responsible, at least in part, for the developmental regulation of the cellular Ela-like activity.

INTRODUCTION

The orderly development of the mammalian embryo depends upon strict spatial, temporal and cell type-specific control of transcription. For RNA polymerase II such control is often exerted at the initiation step and depends on cis-acting DNA sequences normally located upstream of the initiation site. These sequences, which are functionally grouped into either enhancers or promoters, contain motifs which bind trans-acting protein factors which modulate polymerase activity and it is the combinatorial action of such sequence-specific DNA binding proteins which confers on a given gene its particular pattern of transcriptional activity (1, 2). We wish to define transcription factors that function during murine early embryogenesis and which are themselves regulated as differentiation proceeds. We have approached this problem by studying transcriptional regulation in embryonal carcinoma (EC) stem cells which, although derived from teratocarcinomas, share a number of properties with early embryonic cells (3). Of particular importance is the fact that EC cells can be induced to differentiate in vitro into a variety of cell types (4) thus allowing a systematic analysis of the mechanisms that regulate gene expression during embryonic cell commitment and differentiation.

Studies employing both DNA-mediated gene transfer into EC stem cells and their differentiated derivatives, and in vitro transcription systems derived from such cell types, have revealed some interesting features of transcriptional regulation in stem cells. Imperiale et al. (5) showed that the adenoviral early E2A promoter, which is trans-activated by viral Ela in infected cells (6), is expressed more efficiently in F9 EC stem cells infected with dl312, a deletion mutant which lacks the activating Ela gene product (7), than in either F9 cells differentiated to parietal endoderm (F9-PE) or the differentiated cell line Hela, suggesting the existence of a cellular activity that can complement viral Ela.

We have previously shown that this activity can be reproduced in in vitro transcription extracts and that the early E2A promoter binds proteins in stem cell extracts which are present at reduced levels in differentiated cell extracts (8).

Some viral enhancers are either poorly active or exert a negative effect in EC stem cells (9–13). Since adenoviral Ela gene products also trans-repress enhancers (14, 15) and polyoma enhancer mutants which function in stem cells are refractory to this repression (16) it has been suggested that the enhancer repression observed in stem cells is also due to the cellular Ela-like activity. However, this is unlikely to completely explain this phenomenon since our in vitro analyses of SV40 enhancer function suggest that its inactivity in stem cells is mainly due to the lack of the requisite positively acting factors (17), a view supported by the data of Kryske et al. (18) which show that PEA1, a factor required for polyoma enhancer function, is present at low levels in stem cells but is activated upon differentiation.

In the present study we define trans-acting protein factors that bind to DNA sequences within the E2A promoter. Proteins which can bind to the distal, CRE-containing element are present in both EC cells and differentiated derivatives. Another binding activity, termed DRTF1, functions as a positively acting...
transcription factor in F9 EC cell extracts and is down regulated as these cells differentiate to F9-PE. Our data suggest a role for both CRE-binding proteins and DRTF1 in mediating the cellular Ela-like activity.

MATERIALS AND METHODS

Eukaryotic cells

All cells were grown as adherent monolayers in Dulbecco's modification of Eagle's medium containing 10% (v/v) foetal calf serum and antibiotics. F9 EC cells were grown and differentiated as described previously (8). HeLa cells were infected or mock infected for 8h as previously described (19).

Cell extracts and in vitro transcription

Whole cell extracts, in vitro transcription and primer extension were performed exactly as previously described (8). Nuclear extracts were prepared from infected and mock infected HeLa cells as previously described (19).

Recombinant plasmids and probes

pE2Aco/ contains the Ad5 E2A promoter from —96 to +68 and was digested with BglII and HindIII to yield the wild-type promoter fragment. pEC -17 was kindly provided by Dr. J. Nevins and has been described previously (20). LS —63/-52 (21) was purified after digesting with BglII and Hindm. All probes were purified from LGT agarose and end-labelled by conventional procedures.

Oligonucleotides

Oligonucleotides were deprotected by incubating in ammonia at 55°C for 8h, and ethanol precipitated. Oligonucleotides were radiolabeled using T4 polynucleotide kinase and y-32P-ATP by conventional procedures.

Gel retardation

Gel retardation assays were performed as previously described (8), except that the non-specific competitor was sonicated salmon sperm DNA. Typically binding reactions contained 4.0/µg cell extract and 2.0/µg sonicated salmon sperm DNA preincubated for 10 min at 30°C, followed by another 10 min at 30° after addition of approximately 0.5ng probe. Complexes were loaded onto a 4% Tris-acetate-EDTA (pH 8.3) gel and electrophoresed for 90 min at 150V.

DNase footprinting

The exact conditions for DNase footprinting were determined for each extract and probe examined. Binding reactions usually contained about 50µg EC stem cell extract, 2µg non-specific competitor and 0.5ng end-labelled or kinase-labelled probe. Incubations were performed as for the gel retardation analysis after which MgCl2 and CaCl2 (final concentrations 5mM and 2.5mM, respectively) and DNase 1 (1.0 to 10.0µg/ml) were added and incubated for a further 1 min. at room temperature. The digestion was stopped by the addition of EDTA to a final concentration of 20mM, the reaction mixture was extracted with phenol-chloroform, precipitated and analysed on a 10% acrylamide-urea gel.

RESULTS

Delineation of E2A sequences that bind protein factors in EC stem cell extracts

The Ad5 E2A promoter has been used to define DNA binding activities in F9 EC stem cell extracts which are down regulated following cAMP and retinoic acid treatment for 5 days (8). Figure 1 shows a typical gel retardation analysis with wild-type promoter sequences in which a number of discrete shifts are resolved. We show below that the protein factors that cause these shifts bind sequences -94 to -71 (*) and -71 to -50 (-).

Protein binding sites were defined by DNase 1 footprinting on both the coding and non-coding strands in F9 EC cell extracts. By studying DNase sensitivity as a function of increasing amount of F9 EC stem cell extract we could, with a 3' end-labelled non-coding strand probe, define three protected regions upstream of -30 (Fig. 2a). The most distal region had a 5' border at -82 since the A at this position was clearly protected whereas the C at -84 was not (the G at -83 was not sensitive to digestion and so the precise extent of protection could not be determined). This footprint extended to the G at -73 which, although it had only marginally decreased sensitivity to DNase, is required for efficient binding of this factor (see later), and an increased exposure indicated that the C at -72 was in fact protected, suggesting that this nucleotide marks the 3' boundary of this binding site. A HeLa cell protein called ATF (22,23) binds to this distal E2A promoter region. This sequence also binds CREB, a protein involved in mediating cAMP induction of promoter activity (24). The protein defined in the present study as binding to this region of the promoter is, however, distinct from ATF/CREB according to several criteria including molecular weight analysis (data not shown); we refer to this binding activity as TF68.

The next footprint on the non-coding strand was immediately adjacent to this and protected sequences from —71(A) to and including —62(G). Since the —60(C) position remained sensitive
Figure 2. DNase footprinting analysis of E2A promoter binding sites occupied in F9 EC stem cell extracts. (a) Footprints resolved on the non-coding strand. The E2A promoter fragment (−96 to +68), end labeled at the HindIII site (−96), was incubated in the absence (tracks 2, 3 and 4) or presence (tracks 5, 6, 7 and 8) of increasing amounts of F9 EC cell extract and further treated with either 1µg/ml (track 3) or 10µg/ml (tracks 4, 5, 6, 7 and 8) DNase as described in Materials and Methods. Track 1 shows the G + A sequence ladder. A region showing partial protection (−50 to −35) is indicated by the broken line and the arrow indicates a hypersensitive site. (b) Footprints resolved on the coding strand. The E2A promoter fragment (−96 to +68), kinase-labelled at the HindIII site (−96), was incubated in the absence (tracks 1, 2 and 3) or presence (tracks 4, 5 and 6) of increasing amounts of F9 EC cell extract and further treated with either 1µg/ml (track 2) or 10µg/ml (tracks 3, 4, 5 and 6) DNase as described in Materials and Methods. (c) Footprints resolved on the non-coding strand. The E2A promoter fragment prepared as in (a) was incubated in the absence (tracks 1) or presence (tracks 2, 3 and 4) of increasing amounts of F9 EC cell extract and further treated with either 1µg/ml (track 1) or 10µg/ml (tracks 2, 3 and 4) DNase as described. This autoradiograph shows clearly the position of the DRTF2 binding site. (d) Footprints resolved on the non-coding strand of LS −63/−52. Mutant E2A LS −63/−52 (−96 to +68), end labelled at the HindIII site (−96), was incubated in the absence (track 1) or presence of increasing amounts of F9 EC cell extract (tracks 2, 3 and 4) and further treated with either 1µg/ml (track 1) or 10µg/ml (tracks 2 to 4) DNase as described in Materials and Methods.

A number of other weak footprints are evident in Fig. 2a, for example in the −45 to −30 region, and these have not yet been characterised in detail; we also note a number of hypersensitive sites (Fig. 2a, arrow).

To summarise, three protein factors which bind to the E2A promoter have been characterised in F9 EC cell extracts. The
Figure 3. (a) Distribution and regulation of binding activities. Gel retardation analysis with either probe oct (tracks 1 to 4), probe 94/71 (tracks 5 to 8) or probe 71/50 (tracks 9 to 16) with 4.0μg of either F9 EC (tracks 2, 6, 10 and 14), F9-PE (tracks 3, 7, 11 and 15) or HeLa (tracks 4, 8, 12, and 16) cell extracts. The gel was run for 90 min at 150V and exposed for approximately 16h, apart from tracks 13 to 16 which are an increased exposure (48h) of tracks 9 to 12. A non specific complex (NS) is indicated. (b) Summary of oligonucleotides used in the present study. The top row shows the wild-type E2A promoter sequence under which are the names and sequence of the various oligonucleotides used in this study. Nucleotides in bold represent alterations from the wild-type sequence.
Figure 4. Sequence specificity of DRTF1 (a) Gel retardation with probe 71/50 in either uninfected (tracks 2, 4, 5 and 6) or infected (track 3) HeLa cell extracts in the presence of competing probe 71/50 (track 5, 300ng) or probe 69/64* (track 6, 245ng) and 2.0μg (tracks 2 and 3) or 4.0μg (tracks 4, 5 and 6) cell extract. Track 1 shows the probe alone; NS indicates a non specific complex. (b) Gel retardation with probe 71/50 in F9 EC cell extracts in the presence of competing probe 71/50 (track 2, 300ng), 69/64* (track 3, 245ng), 69/66* (track 4, 270ng), 69/68* (track 5, 290ng), 64* (track 6, 214ng), 63* (track 7, 266ng), 62/60* (track 8, 360ng) or 64/50 (track 9, 440ng). Track 1 shows the complexes resolved in the absence of competitor; NS indicates a non specific complex. (c) Gel retardation with wild-type E2A promoter in F9 EC cell extracts in the presence of competing probes as detailed in (b) and in addition either probe 57/46 (track 8, 180ng), 73/63 (track 9, 140ng), 70/60 (track 10, 140ng), or 68/58 (track 11, 165ng). These gel retardation experiments were exposed for 16h each.

distal footprint protects sequences in the −82 to −70 region and results from binding of TF68. DRTF1 binds to the −70 to −60 region and on the coding strand the 5' border of the footprint merges with that of TF68. DRTF2 binds to the −60 to −50 region and appears to be of lower abundance than DRTF1. These data are summarised in Fig. 8.

Regulation of DNA binding activities during F9 EC cell differentiation

We first investigated the integrity of the cell extracts by studying a DNA binding protein which should be at similar abundance in different extracts. For this purpose we chose to analyse the protein factor referred to as OTF1 (25) which binds to the octamer sequence ATGCAAAT, since there is no reason to believe that the abundance of this protein factor is regulated in different cell types. Such an analysis is shown in Fig. 3a (tracks 1 to 4); the oligonucleotide oct, containing a consensus octamer motif, detected a protein at similar abundance in F9 EC, F9-PE and HeLa cell extracts (arrow). This binding activity was dependent on the octamer sequence since a mutated motif prevented the formation of this complex (data not shown). That this binding activity was similar in the three extracts analysed, argues that the cell extracts were not, for example, prepared with different efficiencies.

To investigate the cellular distribution of the binding activities defined by footprinting, we synthesised a panel of oligonucleotides that represent the putative DNA binding sites. Probe 94/71 contains E2A DNA sequences from −94 to −71 (Fig. 3b) encompassing the distal footprint. When used in the gel retardation this oligonucleotide probe resulted in two shifts in the extracts analysed (Fig. 3a, tracks 5 to 8); the slowest migrating shift was the result of specific binding to probe sequences and the faster migrating shift (marked NS) the result of non specific protein-DNA binding since it could be competed by unrelated DNA sequences (data not shown). Comparison of the specific shift intensity indicated that it was similar in the three extracts analysed, there being perhaps a slightly higher level of the detected factor in differentiated cells. Further analysis of this activity indicated that DNA binding to probe 94/71 requires the CG (−73 and −72) dinucleotide (data not shown).

The abundance of DRTF1 was then analysed using probe 71/50 (sequences −71 to −50, Fig. 3b) containing the DRTF1 footprint site, which bound a multiplicity of different activities in F9 EC cell extracts (Fig. 3a, tracks 10 and 14; a, b and c); shorter DNA sequences, such as 64/50, 57/46, 73/63, 70/60 and 68/58, failed to bind these activities. The abundant shifts (b and c) have similar mobility and characteristics to those formed on the wild-type promoter and in addition probe 71/50 competed efficiently for this promoter shift (Fig. 4c, track 3), arguing that the oligonucleotide binds the same activity as the wild-type promoter. Thus we assume that the footprint encompassing this region results from the same factor, DRTF1.

The complex F9 EC shift pattern had clear differences from
that produced by F9-PE (Fig. 3a, compare tracks 10 and 11 with 14 and 15). In particular, the two abundant shifts (b and c) which migrated as a closely spaced doublet just behind the non specific shift (NS) were much reduced in F9-PE extracts. In contrast, the slower migrating shift (a) was detectable in F9-PE but at lower abundance (Fig. 3a, compare tracks 14 and 15). This shift pattern suggests that the DNA sequence defines a multiplicity of proteins that have similar sequence specificity, and furthermore argues that the protein factors defined by this sequence are quantitatively down regulated as F9 EC cells differentiate to F9-PE but in a non co-ordinated fashion; this is in contrast to O TF1 which undergoes no overt regulation.

Probe 71/50 also bound an activity present in HeLa cell extracts (Fig. 3a, tracks 12 and 16). Close inspection of this shift pattern indicated that the HeLa and F9 EC doublets have subtly different mobility (Fig. 3a, compare tracks 10 and 12) and a quantitative inspection indicated that the F9 EC extract doublet was more intense relative to HeLa cell extracts (approximately 2-fold from densitometer scanning of several retardation experiments). HeLa cell extracts also produced a slower migrating complex (d) which again had mobility distinct from the a-form in F9 EC and F9-PE extracts. To summarise, probe 71/50 defines a multiplicity of DNA binding activities in F9 EC cell extracts which are regulated as these cells differentiate to F9-PE cells. In HeLa cell extracts the shift pattern is different and the factors responsible are at lower abundance than in F9 EC cell extracts.

Sequence specificity of DRTF1

The sequence specificity of DRTF1 was assessed by competing either probe 71/50 (Fig. 4b) or the E2A wild-type promoter (Fig. 4c) with particular oligonucleotides (Fig. 3b). As expected, the homologous sequence (probe 71/50) competed efficiently for 71/50 dependent complexes (Fig. 4b, track 2) whereas at similar concentration the mutation 69/64* did not (Fig. 4b, track 3), indicating that the sequence —69 to —64 is necessary for this competition and hence DRTF1 binding. Reduced competition was evident as this mutated region was reduced such that 69/66* (mutation from —69 to —66) competed poorly (Fig. 4b, track 4) whereas 69/68* (mutation in —69 and —68) competed just about as efficiently as the wild-type sequence (Fig. 4b, compare tracks 5 and 2). This series of mutations competed similarly for the wild-type promoter binding activity such that 69/64* could not compete whereas 69/68* competed as efficiently as probe 71/50. The mobility of the shifts competed off the wild-type promoter with 71/50 (Fig. 4c, track 3) had a similar relative mobility to those bound directly by the oligonucleotide (Fig. 4b, track 1). The wild-type promoter shift that remained post competition with 71/50 resulted from TF68 (Fig. 4c, track 3).

Interestingly, probe 64* competed as efficiently as wild-type 71/50 (Fig. 4b, tracks 2 and 6), indicating that nucleotide —64 can be mutated without affecting the binding efficiency. Mutating other nucleotides in this central region (63* and 62/60*) abolished the binding activity (Fig. 4b, tracks 7 and 8) and again this correlated with the ability of probes 63* and 62/60* to compete with the wild-type promoter (Fig. 4c, track 7 and data not shown).

In summary, the DRTF1 binding site has a 5' border at —67 with the 3' border downstream of —58 and requires some but not all of the GC-rich core sequence.

HeLa cell extracts also contain an activity that bound to probe 71/50, although as already discussed the gel shift pattern was different from that in F9 EC cell extracts. When sequence specificity in HeLa cell extracts was assessed by competition the homologous probe competed efficiently for the specific shift whereas 69/64* did not compete significantly (Fig. 4a, tracks 5 and 6); the remaining oligonucleotide probes competed as in F9 EC extracts (data not shown). This indicates that the HeLa cell activity has similar sequence requirements to the F9 EC activity.

Sequence specificity of DRTF2

Probe 64/50 was used to characterise DRTF2. This probe contains the DRTF2 footprint region (Fig. 2, —60 to —50 region) and importantly did not bind DRTF1 because it could not compete with probe 71/50, which contains the additional sequences (—71 to —65) required to bind DRTF1 (Figs. 4a and c).

In F9 EC cell extracts probe 64/50 gave rise to two specific complexes (Fig. 5, track 2). Comparison of the F9 EC to F9-PE shift pattern indicated that the slow migrating complex (arrow) was down regulated during differentiation. Both these complexes were at low abundance, and we estimate, based on gel retardation, that this activity is approximately 10-fold less abundant than DRTF1. This is consistent with the DNase footprint analysis which showed that the DRTF2 footprint was weaker than that for DRTF1.

The sequence specificity of the slow complex was assessed by competing probe 64/50 with the oligonucleotides used previously to characterise DRTF1. Although probe 64/50 contains sequences from —64 to —50, probe 57/46 failed to compete for complex...
Figure 6 (a) Transcriptional activity of E2A promoter in F9 EC and F9-PE extracts. In vitro transcription was performed as described in Materials and Methods in 20μg of either F9 EC (tracks 1 to 5) or F9-PE (tracks 6 to 10) cell extract with wild-type E2A (tracks 1 and 6, 85ng; tracks 2 and 7, 175ng; tracks 3 and 8, 350ng) or HPRT (tracks 4 and 9, 78ng; tracks 5 and 10, 155ng) promoters. (b) Competition between the E2A promoter and DRTF1 binding site in F9 EC cell extracts. In vitro transcription in F9 EC cell extracts (20μg) with either the wild-type E2A promoter (tracks 1 to 4, 175ng) or the -17 promoter (tracks 5 and 6, 175ng) in the presence of probe 71/50 (tracks 1 and 5, 300ng; tracks 2 and 6, 30ng) or probe 64/50 (tracks 3 and 4, 440 and 44ng, respectively). Tracks 5 and 6 derive from a different experiment employing a primer extension probe of higher specific activity, and should not be taken to imply that the deleted promoter is more active than the wild-type. (c) Competition between the E2A promoter and TF68 binding site in F9 EC cell extracts. In vitro transcription in F9 EC cell extracts (20μg) with wild-type E2A promoter (175ng) in the presence of probe 17/3 (tracks 1 and 2) or 94/71 (tracks 3 and 4) where the amount of competing oligonucleotide was 190ng (track 1), 19ng (track 2), 250ng (track 3) or 25ng (track 4).

formation (Fig. 5, track 5), indicating that sequences -64 to -58 are required to bind DRTF2. That probe 71/50 competed but probe 62/60* did not (Fig. 5, compare tracks 6 and 9) indicates that sequences in the GC rich region (-65 to -60) are also required to bind DRTF2, suggesting that DRTF1 and DRTF2 have overlapping binding sites.

E2A promoter sequences activate transcription in F9 EC stem cell extracts

If the binding activities defined thus far function in transcription then their DNA sequences should activate transcription in vitro. In order to assess this we used our previously developed in vitro transcription assay and compared the transcriptional activity of the E2A promoter in F9 EC and F9-PE cell extracts (Fig. 6a). The hypoxanthine phosphoribosyl transferase (HPRT) gene transcription control sequence was used to control for transcription activity in the extracts, since the expression of this gene is not affected as F9 EC cells differentiate, and as expected it was transcribed equally in F9 EC and F9-PE extracts (Fig. 6a, compare tracks 4 and 5 to 9 and 10). In contrast, the wild-type E2A promoter was more active in F9 EC relative to F9-PE both at low and high template concentration (Fig. 6a, compare tracks 1, 2 and 3 to 6, 7 and 8) indicating that in vitro the activity of E2A resembles the regulated activity in vivo; this is consistent
with the idea that DRTF1 functions as a positively acting transcription factor.

Figure 7. Effect of LS-63/—52 mutation on protein binding in F9 EC and Ad5 infected and uninfected HeLa cell extracts: Gel retardation with either wild-type E2A (tracks 1 to 7) or LS-63/—52 (tracks 8 to 10) in Ad5-infected (tracks 2, 4, 5 and 8) or mock infected (tracks 1, 3, 6 and 9) HeLa cell extracts and F9 EC cell extract (tracks 7 and 10). Tracks 3 and 4 show an increased exposure of tracks 1 and 2, and were electrophoresed for 2.5h instead of 1.5h (tracks 5 to 10). A number of complexes induced in infected cells are indicated by arrows (track 4), and the DRTF1 promoter complex in F9 EC cell extracts is indicated by an arrow in tracks 7 and 10.

Whether TF68 functions as a transcription factor was also assessed using this assay. Probe 94/71, containing the TF68 binding site, competed efficiently for E2A dependent transcription (Fig. 6c, tracks 3 and 4) whereas in the same experiment a control oligonucleotide containing sequences from —17 to +3 (probe 17/3) failed to compete at a similar concentration (Fig. 6c, tracks 1 and 2). These data indicate that DRTF1 and TF68 function to activate transcription in a binding site dependent manner in F9 EC cell extracts.

Relationship of DRTF1 to E2F
Kovesdi et al. (26) and Siva Raman and Thimmappaya (27) have reported that a HeLa cell protein called E2F, present at very low levels in uninfected HeLa cells, is greatly increased in binding activity upon wild-type adenovirus infection. E2F binds to the consensus sequence TTTCGCGC (1, 28) which occurs twice in the E2A promoter, in opposite orientation at positions —68 to —60 and —43 to —36; both sites are equally occupied during infection (27, 29). Moreover, Reichel et al. (30) reported that F9 EC stem cells contain high levels of this factor which is almost undetectable in F9-PE cells; they concluded that the Ela-like activity of F9 EC cells is due to high levels of E2F.

Since DRTF1 binds to a sequence that includes the distal E2F site (—68 to —60) it was important to investigate the relationship between DRTF1 and E2F. Several properties of DRTF1 already argue against exact identity. Firstly, in probe 71/50 the —64 nucleotide could be mutated without significantly affecting the binding of DRTF1 (Fig. 4b, track 6), in contrast to the effect
of methylating this site on infected cell E2F binding (27, 29). It is also clear that E2F requires both of the GC-rich repeats (−68 to −60 and −43 to −36) because one is insufficient for binding (27), again in contrast to the binding properties of DRTF1. Importantly, E2A LS −63/−52, which has sequences −63 to −52 mutated, can not bind infected cell E2F (27). However, the DRTF1 footprint was not altered by this mutation when compared to the wild-type promoter (compare Figs. 2a and 2d), and in contrast the −60 to −50 region footprint was no longer detectable.

Secondly, the cellular distribution of DRTF1 highlights a number of differences. A detectable level of DRTF1-like activity was present in uninfected HeLa cell extracts, a cell-type reported by several groups to contain little E2F activity (26, 27, 31). Furthermore, extracts prepared from infected (8h post infection with adenovirus type 5) HeLa cells contained several discrete shifts produced in infected cell extracts that were either quantitatively induced or qualitatively specific for infected cell compared to uninfected cell extracts (Fig. 7, tracks 1 to 4, arrows indicate induced complexes), suggesting that numerous forms of E2F exist in infected cells and indicating that infected cell-specific complexes could be detected in these experimental conditions. That these infected cell-specific complexes were related to E2F was supported by studying binding to LS −63/−52, which, in agreement with previous studies, failed to bind any of the infected cell induced activities (Fig. 7, compare tracks 5 and 6 with 8 and 9). In fact, LS −63/−52 produced a similar pattern in both infected and uninfected cell extracts (Fig. 7, compare tracks 8 and 9). In F9 EC cell extracts the binding of DRTF1 was somewhat reduced by mutation LS −63/−52 (Fig. 7, compare tracks 7 and 10, arrow), in contrast to the dramatic effect this mutation had on the binding activities in infected cell extracts, again highlighting a difference between DRTF1 and E2F.

Finally, probe 71/50, which binds DRTF1 in F9 EC cell extracts, and contains an E2F binding site, was studied in infected and uninfected HeLa cell extracts (Fig. 4a, tracks 2 and 3). There was little difference in binding activity between these two extracts, in contrast to the situation when the wild-type promoter was used, indicating that probe 71/50 is incapable of binding infected cell forms of E2F and therefore that the DRTF1 activity defined in F9 EC cell extracts is not an infected cell form of E2F.

**DISCUSSION**

Embryonal carcinoma stem cells share a number of properties with early embryonic stem cells (3). They can be induced to differentiate in vitro into a variety of cell-types which in some cases resemble those present in the early embryo. Such differentiation is presumably accompanied by the transcriptional inactivation and activation of particular genes required to maintain either phenotype. Studying this system therefore allows the mechanisms that control gene expression during embryonic stem cell differentiation to be explored.

Numerous studies suggest that F9 EC stem cells contain a transcription activity with similar properties to the trans-activating function of viral Ela (5, 8). Since this cellular Ela-like activity is regulated as EC stem cells differentiate it has been suggested that it could also be responsible for regulating cellular genes. In the present study we have begun to characterise this activity at the molecular level using the adenovirus E2A promoter because during lytic infection of differentiated cells it requires viral Ela gene products for transcriptional activation, a requirement that can also be provided by an F9 EC cellular activity (5). Moreover, because this activating function is lost as F9 EC stem cells differentiate, the transcription factors that mediate this effect should be similarly regulated. Since such proteins must inevitably be involved in controlling the transcriptional activity of cellular genes we have sought to characterise these factors in greater detail and in this study we have shown that the E2A promoter binds several different sequence specific DNA binding proteins and have characterised their regulation during EC stem cell differentiation. Two factors, called DRTF1 and DRTF2, are down regulated as F9 EC cells differentiate whereas the distal, CRE-containing binding site can be occupied in both stem cell and differentiated cell extracts; these data are summarised in Fig. 8.

The distal E2A promoter domain binds a trans-acting factor in F9 EC cell extracts

The distal region of the E2A promoter, including the CRE, binds a protein factor called TF68, and is also recognised by proteins present in differentiated cell extracts. Oligonucleotide competition experiments show that this factor is required for transcription in vitro. The CRE motif can confer cAMP inducibility onto a promoter, a phenomenon thought to be mediated by a CRE-binding protein (CREB) the activity of which is regulated through phosphorylation by cAMP responsive protein kinase A (24). This motif also occurs in a number of adenovirus promoters (23, 32) consistent with their induction by cAMP in some cell-types (33). However, in HeLa cells the CRE is also required for constitutive promoter activity both in vivo and in vitro where it binds a
sequence specific protein of about 45,000 molecular weight, called ATF(22, 23, 34–36); the relationship of TF68 to ATF/CREB is presently unclear.

**DRTF1 and DRTF2 are differentiation-regulated transcription factors**

The DRTF1 binding activity is strongly down regulated as F9 EC cells differentiate, correlating with the transcriptional activity of the promoter both in vivo and in vitro. This activity consists of a multiplicity of DNA binding activities which are both qualitatively and quantitatively different to a similar binding activity in HeLa cell extracts. That the activity of the E2A promoter in F9 EC and F9-PE cell extracts was similar to the activity in vivo is consistent with both TF68 and DRTF1 acting as binding site-dependent positively-acting transcription factors, an idea supported both by in vitro transcription competition experiments performed with these binding sites and by the results of transient transfection assays with appropriately mutated promoter sequences (Murray et al., manuscript in preparation).

The DRTF2 binding activity was also regulated during F9 EC cell differentiation, although competing with the DRTF2 binding site did not affect transcription suggesting that this particular protein has some role other than functioning to positively regulate E2A in F9 EC extracts. Interestingly, the binding sites for DRTF1 and DRTF2 overlap since both require the GC-rich region, and it will obviously be interesting to determine if there is any cross regulation of binding activity by either factor.

Another factor, called E2F, binds a similar DNA sequence to DRTF1. During adenovirus infection of HeLa cells this factor accumulates to high levels (26, 27, 31) and because it requires E2A in F9 EC extracts. Interestingly, the binding sites for DRTF1 and DRTF2 overlap since both require the GC-rich region, and it will obviously be interesting to determine if there is any cross regulation of binding activity by either factor.

The cellular Ela-like activity

The cellular Ela-like activity was originally defined by the ability of F9 EC cells to complement d1312 (5). In this paper we have defined some of the protein factors that mediate this effect and show that at least two transcription factors are involved, TF68 and DRTF1. That the abundance of DRTF1 is influenced by differentiation suggests that this factor functions in the cellular Ela-like activity and goes some way towards a molecular explanation of the regulated activity. Indeed, mechanistically the activation of the E2A promoter in EC and adenovirus infected cells is analogous since both situations involve an activity that binds the CRE (TF68 or ATF/CREB) that acts together with a regulated factor (DRTF1 or E2F) that in the case of DRTF1 may respond to a cellular activity that mimics viral Ela or in the case of E2F responds to viral Ela gene products per se. Characterising this cellular Ela-like activity should help understand transcriptional control in early embryonic stem cells.

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