E1A Functions as a Coactivator of Retinoic Acid-Dependent Retinoic Acid Receptor- β 2 Promoter Activation

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The retinoic acid (RA) receptor (RAR) β 2 promoter is strongly activated by RA in embryonal carcinoma (EC) cells. We examined this activation in the P19 EC-derived END-2 cell line and in E1A-expressing counterparts and found strong RA-dependent RAR β 2 promoter activation in the E1A-expressing cells, which was not observed in the parental cell line, indicating a possible role for E1A in RAR β 2 activation. In transient transfection assays, E1A functioned as a coactivator of RA-dependent RAR β 2 promoter activation and, moreover, was able to restore this activation in cells lacking RAR β 2 activation. By deletion analysis, two regions in the RAR β 2 promoter were identified that mediate the stimulatory effect of E1A: the RA response element and TATA box-containing region and a more up-stream region between -180 and -63, in which a cAMP response element-related motif was identified as a target element for E1A. In addition, determination of endogenous E1A-like activity by measuring E2A promoter activity in transient transfection assays in EC and differentiated cells revealed a correlation between RA-dependent RAR β 2 promoter activation and the presence of this activity, suggesting an important role for the cellular equivalent of E1A in regulation of the RAR β 2 promoter. (Molecular Endocrinology 7: 604–615, 1993)

INTRODUCTION

Retinoids are known to play an important role in many biological processes, such as cell growth, differentiation, and development (1–3). One of the most striking effects of the retinol metabolite retinoic acid (RA) is its ability to induce differentiation in embryonal carcinoma (EC) cells. The murine P19 EC cell line, for example,

which shares many characteristics with the pluripotent inner cell mass cells of the early mouse embryo, is able to differentiate into a variety of cell types upon the administration of RA (4, 5). The biological action of RA is mediated by two specific sets of nuclear retinoid receptors, RA receptors (RARs) and retinoid X-receptors (RXRs), which belong to the multigene family of steroid/thyroid hormone receptors (for reviews, see Refs. 6–8). These receptors function as ligand-inducible transacting transcription factors and modulate the expression of target genes by interaction with specific *cis*-acting DNA elements, the hormone response elements. Both subfamilies of retinoid receptors consist of several receptor subtypes, referred to as RAR α , - β , and - γ (9–14) and RXR α , - β , and - γ (15–17). Recent studies have identified 9-*cis* RA as the natural ligand for RXRs (18, 19).

The three RAR subtypes were found to be distinctly and spatio-temporally expressed during mouse embryogenesis (20–23), suggesting a specific role for each subtype in development. In addition, different isoforms of each RAR subtype have been identified, which differ in their N-terminal A region (13, 24–26). The mRNAs of these isoforms are generated by differential promoter usage and alternative splicing. For RAR β , for example, three mouse mRNA isoforms (mRAR β 1, - β 2, and - β 3) have been found that are transcribed from two different promoters in the same gene (26). The most up-stream located promoter is used for the transcription of mRNA β 1 and - β 3, which are predominantly expressed in fetal and adult brain and thought to play a role in the development of the central nervous system. RAR β 2 mRNA, transcribed from the other promoter, is highly expressed in RA-treated EC and embryonic stem cells, which suggests that this isoform may be important during early stages of development (26).

In murine EC cells, RAR α and - γ are constitutively expressed, whereas the RAR β gene is rapidly and strongly induced after RA treatment (27, 28). This induction is due to transcriptional activation of the RAR β 2 promoter, which contains a RARE positioned five nu-

cleotides in front of the TATA box (29, 30). The expression of other genes, such as the laminin-B1 gene, is altered later during RA-induced differentiation of EC cells, generally some 24–48 h after RA addition (31). The promoter of the laminin-B1 gene was also found to contain a RARE, although different from the RARE identified in the RAR β 2 promoter and with a lower affinity for RARs (32). Interesting in this respect is the identification of the so-called coregulators, proteins that are able to associate with RARs and thereby modulate their affinities for different RAREs (33). In fact, members of the RXR subfamily have been recently identified as coregulators of RAR action (17, 34–36). Adenovirus E1A products are required for transactivation of adenovirus early genes.

The role of E1A in cellular transformation has been well studied (for reviews, see Refs. 37 and 38). E1A alone has the property of immortalizing primary cells in culture (39, 40), and in cooperation with the adenoviral E1B gene or an activated *ras* gene is capable of fully transforming cells (40, 41). The E1A transcription unit produces two major spliced mRNAs, the 12S and 13S forms, which encode proteins consisting of 243 and 289 amino acids, respectively. These proteins are able to activate or repress the transcription of both cellular and viral genes (37, 42–45). Three regions (CR1–3) have been identified in the E1A 13S protein, which are highly conserved among the E1A proteins from different adenoviruses. The E1A 12S protein contains CR-1 and CR-2, which are required for cellular transformation, but lack CR-3, which is known to be necessary for transcriptional activation of viral early genes (for reviews, see Refs. 38 and 46). In general, the 13S E1A product is involved in the transactivation of target genes, while the 12S protein functions as a repressor.

The molecular mechanism underlying gene regulation by E1A is still not completely understood. Studies to unravel this mechanism have been made complicated by the failure to show that E1A could bind to specific DNA sequences (47, 48). However, analyses of the promoter regions of E1A-inducible genes lead to the identification of a DNA element involved in mediating the effect of E1A on transcription. This element appeared to be identical to the cAMP response element (CRE) and binds the cellular transcription factor ATF or CRE-binding protein (CREB) (for review, see Ref. 38 and references therein). In addition, for several genes, TATA-binding factors were found to play an important role in mediating the transactivation by E1A (43, 49–51). Apart from interactions with DNA-binding proteins, E1A was also shown to interact with other cellular proteins, among them the retinoblastoma gene product (52, 53). These findings lead to the postulation that E1A exerts its function through multiple cellular or viral proteins.

In EC cells, the existence of an E1A-like activity has been reported which is lost upon differentiation with RA (54, 55). This was based on the observation that the adenoviral E2A gene, expression of which requires the E1A proteins, is active in EC cells (42), whereas, on the

other hand, the polyoma virus enhancer, which is repressed by E1A (44, 56), also has reduced activity in undifferentiated cells, but becomes active upon differentiation (57–59). E1A-like activity could also be detected in mouse oocytes and preimplantation-embryos (60, 61). However, the factors that form the basis of this E1A-like activity have yet to be identified. In this report we studied RA-dependent RAR β 2 promoter activation in END-2 cells, a RA-differentiated derivative of P19 EC cells (62), and in END-2 E1A cells, which have been stably transfected with an E1A expression vector (63). In contrast to the situation in END-2 cells, the RAR β 2 gene is strongly activated by RA in the E1A-expressing derivatives, indicating a possible involvement of E1A in RA-induced RAR β 2 promoter activation. To test this hypothesis, we performed transient transfection assays in several cell lines using RAR β 2 promoter-containing reporter constructs and E1A expression vectors. We demonstrate that RA-dependent RAR β 2 promoter activation can be restored by cotransfection of E1A expression vectors in cells in which normally this activation is not observed. In addition, we show that two regions in the RAR β 2 promoter are involved in conferring the stimulatory effect of E1A and RA. Finally, we postulate that the cellular equivalent of E1A is able to function as a coregulator of RA-dependent RAR β 2 promoter activation.

RESULTS

RAR Expression in END-2 and END-2 E1A Cells

P19 EC cells, which form a model system for studying early mouse embryogenesis, are tumorigenic cells and exhibit all of the characteristics of transformed cells, including anchorage-independent growth and proliferation in the absence of growth factors. END-2 cells, cloned from P19 EC cells differentiated by aggregation in the presence of RA, have lost the transformed phenotype and possess endoderm-like characteristics (62). From this cell line, three clones have been isolated that are stably transfected with the adenovirus type 5 E1A gene, designated clones 20, 23, and 24. These clones, which express both E1A mRNA and protein, have regained some characteristics of EC cells and possess a more EC cell-like morphology (63).

We questioned whether the E1A-induced change in differentiation state in END-2 cells is accompanied by an altered expression pattern of RARs. To address this, we cultured END-2 and END-2-E1A cells for 0, 8, or 24 h in the presence of RA and determined RAR expression by RNase protection analyses using RAR α -, β -, and γ -specific complementary riboprobes, as described in *Materials and Methods*. Since these probes are directed against the C/D and E/F regions, all isoforms present will be recognized. Figure 1A shows that both the END-2 and END-2-E1A cell lines contain a similar level of RAR α and γ transcripts in the absence of RA, and these transcript levels are not significantly

Fig. 1. RAR Expression in Untreated and RA-Treated END-2 and END-2 E1A Cell Lines

A, RNA obtained from END-2 cells and three different END-2-E1A clones treated for 0, 8, and 24 h with RA was simultaneously hybridized with RAR α , - β , and - γ - and GAPDH (gap)-specific riboprobes in a RNase protection analysis. As controls, lanes indicated α , β , and γ show the protected mRNA fragments when the RAR α , - β , and - γ riboprobes were hybridized alone to RNA isolated from P19 EC cells treated for 24 h with RA. B, Relative RAR β expression was determined by quantification of the RAR β transcript levels shown in A and correction for the GAPDH transcript levels.

affected by RA treatment. RAR β transcripts could not be detected in untreated END-2 and END-2-E1A cells; however, RA treatment resulted in the induction of RAR β expression. Interestingly, this induction appeared to be stronger in the E1A-expressing cell lines than in the parental END-2 cells (see also Fig. 1B).

RA-Dependent RAR β 2 Promoter Activation in END-2 and END-2 E1A Cells

The strong RA-dependent increase in the amount of RAR β transcripts in END-2 E1A cells, which was observed to a lesser extent in the parental cell line END-2, suggested the possibility that stable expression of E1A affects this induction. We next investigated whether this was due to an enhanced transcriptional activation of the RAR β 2 promoter in END-2-E1A cells compared to the situation found in the parental END-2 cell line. We, therefore, performed transient transfection assays with a chloramphenicol acetyltransferase (CAT) reporter construct containing the -1470/156 fragment of the human RAR β 2 promoter and determined CAT activity in untreated and RA-treated END-2 and END-2-E1A cells. Figure 2 shows a strong RA-dependent increase in CAT activity on this construct in the E1A-expressing clones 20 and 23 and, to a lesser extent, in clone 24, while a much smaller induction is seen in END-2 cells. These results demonstrate that the enhanced level of RA-induced RAR β expression in the E1A-expressing END-2 cells compared with the non-transfected END-2 cells is due to a stronger activation of the RAR β 2 promoter by RA.

The finding that the accumulation of RAR β transcripts after RA treatment in the END-2-E1A cell lines (Fig. 1) is not completely consistent with the observed increases in CAT activity might be explained by differences in transcript and/or protein stability in these cells. In addition, the variation found in the level of activation among the three END-2-derived clones could be due to different levels of E1A proteins expressed in each cell line (63).

Effects of E1A 12S and 13S on RA-Dependent RAR β 2 Promoter Activation in Different Cell Types

As mentioned previously, stable expression of E1A in END-2 cells causes a change in their state of differentiation (63). Therefore, the enhanced RA-dependent RAR β 2 promoter activation observed in END-2-E1A cells could be due to an indirect effect of E1A. To investigate whether E1A affects the RAR β 2 promoter more directly and to examine which E1A protein, the 12S or the 13S form, enhances the level of RA-induced RAR β 2 transcription, we transfected END-2 cells with the -1470/156 CAT construct in combination with 1 μ g DNA E1A expression vectors and performed CAT assays in the absence and presence of RA. Interestingly, after RA treatment, cotransfection of the vector pE1A 13S resulted in an increase in CAT activity, while a small repressive effect was observed when using the pE1A 12S vector (Fig. 3). In the absence of RA, cotransfection of the E1A expression vectors had no effect on the level of CAT activity (not shown). One microgram of E1A expression vector was used in these assays

Fig. 2. RA-Dependent RAR β 2 Promoter Activation in END-2 and END-2-E1A Cells

Comparison of the level of RA-dependent RAR β 2 promoter activation by transient transfection assays in END-2 cells and in the three E1A-expressing derivatives, clones 20, 23, and 24, using a CAT reporter construct containing nucleotides -1470 to 156 of the human RAR β 2 promoter. CAT activity was determined in extracts of untreated and RA-treated cells by determining the percent conversion of [^{14}C]chloramphenicol to its acetylated products. A representative experiment is shown.

because this amount appeared to give the optimal response in dose-response studies (not shown). A vector containing a β -actin promoter-driven CAT gene served as a positive control in the assay.

In addition, we studied the effects of both E1A products on RA-dependent RAR β 2 promoter activation in two other cell lines: the human colon tumor cell line HCT116, in which RAR β 2 activation cannot normally be detected (van der Leede, B.-j. M., in preparation), and P19 EC cells, which show strong RA-dependent RAR β 2 promoter activation. Therefore, the CAT assays performed in END-2 cells were repeated in these cell lines. As shown in Fig. 3, in the absence of E1A expression vector, the RAR β 2 promoter is insensitive to the inducing effect of RA in HCT116 cells. However, cotransfection of pE1A 13S enables the promoter to be activated by RA, whereas cotransfection of the pE1A 12S vector has no effect.

In P19 EC cells, the E1A 12S product has a repressive effect on RA-induced CAT activity, while the 13S product leads to even stronger activation of the RAR β 2 promoter by RA (Fig. 3).

From these results it can be concluded that dependent on the cell lines used, 12S E1A has no or a repressive effect on RA-dependent RAR β 2 promoter activa-

tion, while 13S E1A can stimulate and in other cases can restore this activation.

Determination of E1A-Sensitive Regions in the RAR β 2 Promoter

We next examined which sequences of the RAR β 2 promoter are involved in conferring the stimulatory or repressive effect of E1A in transient transfection assays in the different cell lines.

First, END-2-cl23 cells were transfected with several CAT reporter constructs containing 5'-progressive deletions of the $-1470/156$ RAR β 2 promoter fragment (Fig. 4A), and their activation by RA was determined. Figure 4B shows that constructs containing the sequences between -180 and 156 have comparable levels of activation, while a 3- to 4-fold decrease in CAT activity is observed when the RAR β 2 promoter fragment encompassing nucleotides -180 to -63 is removed. In addition, the RARE-containing $-63/156$ fragment is sufficient for mediating RA-dependent activation.

Secondly, we transfected these deletion constructs in combination with the pE1A 13S or pE1A 12S vector in HCT116 cells (Fig. 4C). As described above for HCT116 cells, 12S E1A had hardly any effect on RAR β 2 promoter activation. Using pE1A 13S, the levels of induction as measured on the $-180/156$ construct appeared to be similar to the levels found when the $-1470/156$ reporter was used. However, a 3-fold lower level of RA-induced CAT activity was observed on the $-63/156$ construct. As controls, expression of the 12S and 13S products in HCT116 cells in the absence of RA resulted in only a slight increase in the basal level of transcription in the case of 13S E1A.

Finally, in P19 EC cells, the $-180/156$ and $-63/156$ CAT constructs were tested for activation by RA, while the amount of cotransfected pE1A 12S and pE1A 13S expression vector was varied. Figure 4D shows that the E1A 13S product is able to enhance expression of the already strongly activated RAR β 2 promoter by 50–70% when 1.25 or $2.5 \mu\text{g}$ pE1A 13S was cotransfected with the $-180/156$ reporter construct. Transfection of higher amounts of pE1A 13S did not lead to higher levels of induction (not shown). In contrast, cotransfection of the pE1A 12S expression vector with the same reporter construct resulted in an approximately 50% reduction in RA-induced RAR β 2 promoter activation compared to the normal level of induction. Interestingly, when using the $-63/156$ reporter construct, the E1A 13S product had a repressive effect on activation of the promoter by RA, which was of the same order as the repression found with the E1A 12S product on this reporter (Fig. 4D).

Taken together, the deletion studies in the E1A stably transfected END-2-cl23 cell line and the cotransfection experiments in HCT116 and P19 EC cells show that two regions in the RAR β 2 promoter mediate the effects of E1A and RA: the RARE containing $-63/156$ promoter fragment and sequences located between -180

Fig. 3. Comparative Analyses of RA-Dependent RAR β 2 Promoter Activation in END-2, HCT116, and P19 EC Cells in CAT Assays upon Cotransfection with E1A Expression Vectors

END-2, HCT116, and P19 EC cells were transfected with the -1470/156 reporter construct alone or in combination with an E1A 12S or 13S expression vector, and CAT activity was determined in the presence or absence of RA. A representative experiment is shown. A β -actin-CAT reporter plasmid served as a positive control in the assay (C).

Fig. 4. Localization of RAR β 2 Promoter Regions Mediating E1A and RA-Dependent Activation

A, Schematic representation of the human RAR β 2 promoter-derived 5'-progressive deletion CAT constructs. The relative positions of the up-stream stimulatory promoter region (■), the RARE, the TATA box, and the 5'-ends of the reporter constructs used in the experiments are indicated. B, CAT assays with the indicated reporter constructs in untreated and RA-treated END-2-cl23 cells. A representative experiment is shown. C, Cotransfection assays in the human colon tumor cell line HCT116 with 1 μ g E1A 12S or 13S expression vector in combination with the indicated reporters. The mean $\pm$ SD of three independent experiments are shown. D, Cotransfection assays with the indicated reporter constructs and varying amounts of E1A expression vectors in P19 EC cells. The level of RA-induced CAT activity is depicted as a percentage of the level of activity normally found in the absence of cotransfected E1A expression vectors (control). The mean $\pm$ SD of three independent experiments are shown.

and -63. Furthermore, the cotransfection experiments demonstrate that 13S E1A exerts its action via both promoter regions, while the repressive effect of E1A 12S on RA-induced RAR β 2 promoter activation is mediated by sequences located on the -63/156 fragment. However, there appear to be cell-specific factors able to modulate the effect of E1A on RAR β 2 promoter activation, because the E1A 13S product functioned as a repressor in P19 EC cells on reporter constructs lacking the -180/-63 promoter fragment, whereas in HCT116 cells, activation was restored.

Identification of a CRE-Like Sequence as E1A Response Element

In earlier studies in P19 EC cells, we found that besides the RARE, more up-stream located sequences are involved in both mouse and human RAR β 2 promoter activation by RA (28, 64). Moreover, we have recently identified an element, located at position -99 to -92 in the RAR β 2 promoter, that enhances RA-dependent promoter activation (65). This element, designated CRE- β 2, is conserved in both the human and mouse RAR β 2 promoter and consists of the motif TGATGTCA, which closely resembles the CRE consensus TGACGTCA. We demonstrated that CRE- β 2 can bind members of the ATF/CREB transcription family (for review, see Ref. 66) by competition experiments in gel retardation assays using consensus CRE-containing oligonucleotides (65). A specific member, ATF-2, has been reported to mediate transcription activation by E1A (67). Besides CRE- β 2, a second putative regulatory element was localized on position -84 to -78 with homology to the TPA response element (TRE) or AP1-binding site (68, 69). However, we could not find a role for this element, designated TRE- β 2, in RA-dependent RAR β 2 promoter activation in P19 EC cells.

To examine whether these elements are involved in mediating the stimulatory effect of E1A on the -180/-63 RAR β 2 promoter fragment, HCT116 cells were transfected with the -180/156 reporter construct in which either CRE- β 2 (Δ CRE) or TRE- β 2 (Δ TRE) was mutated, and CAT activity was determined in the absence or presence of E1A expression vectors and RA. As observed earlier, no significant increases in CAT activity could be detected after RA treatment and/or cotransfection with pE1A 12S on any of the reporter constructs used (Fig. 5). Interestingly, upon cotransfection with pE1A 13S, similar levels of RA-induced CAT activity were found on the -63/156 and Δ CRE reporter constructs, while the wild-type -180/156 construct mediated a 3-fold higher level of CAT activity. Mutation of TRE- β 2 did not affect the responses of the -180/156 promoter fragment to E1A 13S and RA. These results demonstrate that CRE- β 2 mediates the stimulatory effect of E1A 13S on the -180/-63 region upon RA-dependent RAR β 2 promoter activation.

E1A Enhances Protein Binding to the -180/-63 RAR β 2 Promoter Region

We next questioned whether there are differences in nuclear protein binding to the -180/-63 RAR β 2 pro-

moter region in P19 EC, END-2, and END-2-cl20 cells. To assess this, we performed gel retardation assays with 32 P-labeled -180/-63 promoter fragment and nuclear extracts from these cells cultured in the presence or absence of RA. As shown in Fig. 6, predominantly two protein-DNA complexes, named 1 and 3, were found in nuclear extracts from untreated P19 EC cells. In extracts from P19 EC cells treated for 24 h with RA, an additional protein-DNA complex, called 2, could be clearly detected. In addition, upon longer periods of film exposure to the polyacrylamide gel used for the gel retardation assay, it appeared that a low amount of complex 2 is present in extracts from untreated P19 EC cells (results not shown). Binding complexes 1 and 3 were found in END-2 cell nuclear extracts, while complex 2 could not be detected in either untreated or RA-treated cells. The apparent increase in the amount of complexes 1 and 3 formed after RA treatment, as shown in Fig. 6, is due to a lower protein content of the extracts derived from untreated END-2 cells used in this experiment and is normally not observed. All three complexes were found in nuclear extracts from untreated and RA-treated END-2-cl20 cells, and protein binding to the -180/-63 promoter region using these extracts appeared to be stronger than that in the extracts derived from P19 EC and END-2 cells, which normally show similar levels of complex 1- and 3-binding activities.

Competition experiments using nuclear extracts from RA-treated END-2-cl20 cells showed the specificity of the three binding complexes; binding activity was almost completely lost when using an excess of cold probe, and nonspecific competition with a RARE- β 2 containing oligonucleotide did not reduce binding. Interestingly, CRE- β 2-containing oligonucleotides specifically competed for complex 2-binding activity when using nuclear extracts from END-2-cl20 and P19 EC cells (Fig. 7 and data not shown). TRE- β 2 oligonucleotides did not compete for any of the complexes (not shown). These findings suggest that there are additional, yet unidentified, protein-binding sites localized on the -180/-63 RAR β 2 promoter fragment.

Taken together, these results show that stable expression of E1A in END-2 cells increases the binding of complex 1- and 3-binding activities to the -180/-63 RAR β 2 promoter region and, moreover, induces protein binding to CRE- β 2. Furthermore, RA treatment of P19 EC cells enhances CRE- β 2 binding, which was not observed in END-2- and END-2-cl20 cells.

Correlation between RA-Dependent RAR β 2 Promoter Activation and the Presence of Cellular E1A

The results presented above show that E1A can act as a coactivator of RA-dependent RAR β 2 promoter activation. Since EC cells are thought to contain a cellular equivalent of E1A which is lost upon differentiation, we postulate that the strong activation of the RAR β 2 promoter by RA in P19 EC cells could be facilitated by this

Fig. 5. A CRE-Related Sequence in the RAR β 2 Promoter Is Responsive to E1A

Comparison of the level of RA-dependent RAR β 2 promoter activation on the -180/156, ΔCRE-, ΔTRE-, and -63/156 CAT constructs upon cotransfection with 1 μ g pE1A 12S or 13S in HCT116 EC cells. ΔCRE/CAT was generated by altering the CRE-related sequence TGATGTCA [wild type (WT)] at position -99 to -92 in the -180/156 construct into AGATGTTA by site-directed mutagenesis. In a similar fashion, ΔTRE/CAT was constructed by mutation of TRE- β 2, TGGGTCA at position -84 to -78, into GTGGTCA. The mean $\pm$ SEM of four independent experiments are shown.

Fig. 6. Stable Expression of E1A in END-2 Cells Enhances Protein Binding to the -180/-63 RAR β 2 Promoter Region

Gel retardation assays with nuclear extracts (N.E.) from P19 EC-, END-2-, and END-2-cl20 cells cultured in the absence or presence of RA (24 h) using radioactive labeled -180/-63 RAR β 2 promoter fragment as a probe. The three predominant protein-DNA complexes formed are numbered 1, 2, and 3, and a nonspecific band is indicated by an asterisk. Competition (comp.) experiments (100-fold excess) were performed with cold -180/-63 fragment (specific) or CRE- β 2 (CRE-) and RARE- β 2 (RARE-) containing oligonucleotides.

Fig. 7. Detection of E1A-Like Activity in Several Cell Lines by Determination of E2A Promoter Activity

The E2A promoter containing vector p2CAT and a vector containing a β -actin promoter-driven CAT gene were transiently transfected in the indicated cell lines. The activity of the E2A promoter was related to the activity of the β -actin promoter in the same cell line. The horizontal line represents the level at which E2A promoter activity equals β -actin promoter activity. As a control, p2CAT was cotransfected with the E1A expression vector pE1A 13S (+ E1A). The ratio indicated is the mean of three independent experiments.

endogenous E1A-like factor. In addition, the weak response or even the insensitivity of the promoter to RA in differentiated (END-2 and HCT116) cells could then be explained by assuming that these cells contain only low amounts of this factor or are devoid of it.

To determine whether there is a correlation between RA-dependent RAR β 2 promoter activation and the presence of an endogenous E1A-substituting activity,

we monitored E1A-like activity in the P19 EC, END-2, and HCT116 cell lines. Therefore, these cells were transfected with a CAT reporter construct containing the E1A-dependent E2A promoter, the activity of which reflects the presence of E1A-like activity, as has been demonstrated recently (70). Interestingly, in P19 EC cells, the E2A promoter appeared to be as active as in the E1A-expressing human fetal kidney cell line 293

(71) (Fig. 7). In this cell line, the RAR β 2 promoter can also be strongly activated by RA (not shown). In END-2-E1A-cl23 cells, strong E2A promoter activity was observed, while much lower levels of activity were detected in the END-2 and HCT116 cell lines (7% and 5%, respectively, of that in 293 cells). Cotransfection of the expression vector pE1A 13S resulted in an increase in E2A promoter activity, most profoundly in the END-2 and HCT116 cell lines, and to a lesser extent in the E1A-expressing cell lines and P19 EC cells.

These data indicate the presence of a high level of E1A-like activity in P19 EC cells and much lower levels in END-2 and HCT116 cells, supporting our hypothesis that this factor functions as a coactivator of RA-dependent RAR β 2 promoter activation.

DISCUSSION

RA-induced differentiation of P19 embryonal carcinoma cells is accompanied by strong transactivation of the RAR β 2 promoter by ligand-activated RARs, mediated by a RARE located in the proximity of the TATA box. We examined RA-dependent RAR β 2 promoter activation in the RA-differentiated P19 EC-derived END-2 cell line (62) and in their E1A-transfected clonal derivatives (63), and found strong activation in the E1A-containing cells that was not observed in the parental cell line (Figs. 1 and 2). This result prompted us to investigate whether E1A can function as a coactivator of RA-dependent RAR β 2 promoter activation. A comparative analysis of promoter activation in END-2, HCT116, and P19 EC cells by transient transfection assays using E1A expression vectors showed that the E1A 13S product enhances or restores RAR β 2 promoter activation in these cell lines, while the E1A 12S product either has no or a repressive effect (Fig. 3). In both END-2-E1A stable transfectants and cotransfection experiments in P19 EC and HCT116 cells, deletion analysis of the RAR β 2 promoter revealed the presence of two E1A-responsive regions; a 117-basepair (bp) fragment between -180 and -63 and a fragment that contains the RARE and the TATA element (Fig. 4). In addition, a CRE-related sequence at position -99 to -92 in the 117-bp fragment was identified as an E1A response element (Fig. 5). Protein binding studies to the -180/-63 promoter region in gel retardation experiments with nuclear extracts from different cell lines showed that CRE- β 2-binding activity is enhanced after RA treatment in P19 EC cells, which was not the case in END-2 and END-2-E1A cells, and that E1A increases protein binding to the -180/-63 fragment (Fig. 6). Furthermore, examination of endogenous E1A-like activity in P19 EC, END-2, and HCT116 cells by determining the activity of an E2A-promoter/CAT construct (Fig. 7) suggested a correlation between the presence of an E1A-like activity and the response of the RAR β 2 promoter to RA.

Evidence for the Involvement of a Cellular E1A-Like Factor in RAR β 2 Promoter Activation

The reported activation of the RAR β 2 promoter by RA in, for example, CV-1 and COS-1 cells requires additional expression of RARs and is weak compared to the level of activation found in P19 EC cells (72, 73). This indicates that in P19 EC cells, the conditions are optimal for RA-dependent promoter activation. The demonstrated high level of RXR β expression in P19 EC cells, which has been found to function as a coregulator of RAR-dependent promoter activation and increases transcriptional activation by high affinity binding of RAR-RXR heterodimers to RAREs (34), could partially explain the strong RAR β 2 promoter activation in these cells. In addition, the results presented here suggest a role for the cellular equivalent of E1A in activation of the RAR β 2 promoter in these cells, which could function as a coactivator in a similar way as E1A. Moreover, the weak or even absent promoter response to RA in differentiated cells could then be explained by the loss of E1A-like activity upon differentiation. The evidence for this hypothesis is as follows. 1) The examination of the presence of an E1A-like activity in P19 EC, END-2, and HCT116 cells revealed a correlation between the presence of E1A-like activity and RA-dependent RAR β 2 promoter activation. In a recent report it was shown that the hepatoma cell line HepG2 contains a relatively high level of E1A-like activity (74). This finding supports our hypothesis, since the RAR β 2 promoter has been shown to be relatively strongly activated by RA in these cells (11). 2) In END-2 and HCT116 cells, E1A is able to stimulate or even restore promoter activation by RA (Figs. 3 and 4). This suggests that E1A can substitute for the endogenous factor present in EC cells. 3) In P19 EC cells, cotransfection of small amounts of E1A expression vector had a repressive effect on RA-dependent activation, as determined on the -63/156 RAR β 2 promoter/CAT construct (Fig. 4). This can be explained by assuming that P19 EC cells contain an optimal amount of an E1A-like factor, and by additional expression of E1A, other participating factors are titrated out and become rate limiting. Candidates for such participating factors are RARs, RXRs, and TATA-binding factors. Interestingly, when the E1A-sensitive CRE-containing -180/-63 promoter region was included in the reporter construct, the E1A 13S product could enhance activation of the promoter by approximately 50% (Fig. 4). This indicates that the E1A interaction sites of the protein that binds to this promoter region, probably ATF-2, are not saturated by the endogenous E1A-like factor.

Taken together, our results suggest an important role for the cellular equivalent of E1A in the regulation of RAR β 2 promoter activation. Since E1A-like activity has also been detected in mouse preimplantation embryos (60), it is possible that this activity contributes *in vivo* to the spatio-temporal expression of RAR β 2.

Possible Mechanism for E1A-Mediated RAR β 2 Transactivation

We have shown here in both stably transfected cell lines and cotransfection experiments that the -63/156 promoter region alone, which contains the RARE and the TATA element and no other known functional elements, was sufficient to mediate the stimulatory effect of E1A on RA-dependent RAR β 2 promoter activation (Figs. 4 and 5). Recently, Schöler *et al.* (75) reported that Oct-4 and E1A are sufficient for distance-independent activation of the basal transcription machinery. They propose a model in which E1A or its cellular equivalent serves as a bridging factor between Oct-4 and the transcription initiation complex. Our results could be explained in a similar way by assuming that E1A is able to interact with both TATA-binding factors and ligand-activated RARs, thereby facilitating activation of the promoter. Since the RARE is closely positioned to the TATA element, it would be interesting to investigate whether this interaction is distance independent in analogy to the situation found with Oct-4 and E1A (75). In addition, the TATA-binding factor TFIID has recently been reported to be able to interact directly with E1A (76, 77). Whether such interactions are also possible with RARs remains to be proven.

The involvement of a CRE-related sequence located in the -180/-63 region in the enhancement of RAR β 2 promoter activation suggests the participation of members of the ATF/CREB transcription factor family (for review, see Ref. 66) in promoter activation by RA and E1A. A specific member of this family, ATF-2, has been shown to be able to interact with E1A directly (67). It is thought that the interaction of E1A with promoter-bound ATF-2 results in transcriptional activation by the positioning of the E1A-activating region at the promoter (67). Such a mechanism could also account for the observed stimulatory effect of E1A found on CRE- β 2. In addition, more evidence for the involvement of ATF-2 in RA- and E1A-dependent RAR β 2 promoter activation is provided by the fact that CR-3, which is absent in the E1A 12S product, is involved in the interaction with ATF-2 (67), which correlates with and also provides an explanation for our observation that E1A 13S and not 12S enhances promoter activation via CRE- β 2 (Fig. 5).

Besides a possible direct interaction between E1A and the CRE- β 2-binding activity, our results show that stable expression of E1A in END-2 cells increases protein binding to the -180/-63 RAR β 2 promoter region and particularly induces CRE- β 2-binding activity (Fig. 6). However, since in earlier studies we found that the increase in CRE- β 2-binding activity induced by RA in P19 EC cells (see also Fig. 6) did not correspondingly result in an enhancement of CRE- β 2-mediated transcription (65), it is too early to conclude that E1A enhances RAR β 2 promoter activation in a more indirect fashion by increasing CRE- β 2-binding protein levels. Additional studies will be necessary to elucidate more

precisely the manner in which E1A enhances or restores RA-dependent RAR β 2 promoter activation.

We propose a model for the way in which E1A affects RA-dependent RAR β 2 promoter activation, which combines the two mechanisms postulated for E1A-mediated transactivation earlier mentioned; E1A affects RAR β 2 promoter activation by functioning as a bridging factor between RARs or attached coregulators (RXRs) and the basal transcription machinery and enhances promoter activity further by interaction with ATF-2.

MATERIALS AND METHODS

Cells and Plasmids

The cells (END-2, END-2 E1A, P19 EC, HCT116, and 293) used in the experiments were cultured on gelatin-coated dishes in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 containing 7.5% fetal calf serum.

A number of the CAT constructs used in transient transfection assays have been described previously (28). CAT constructs encompassing nucleotides -335 to 156, -180 to 156, and -63 to 156 of the human RAR β 2 promoter were generated by subsequently subcloning a *Dde*I-*Bam*HI, a *Hpa*II-*Bam*HI, and a *Sma*I-*Bam*HI promoter fragment in the promoterless pKTCAT vector.

The -180/156 CAT construct containing either a mutated CRE- or TRE-related sequence was generated by site-directed mutagenesis using the Altered Sites In Vitro Mutagenesis System Kit (Promega, Madison, WI). Briefly, single-stranded DNA derived from the vector pSELECT inserted with the *Hpa*II-*Bam*HI promoter fragment functioned as a template for mutagenesis using the synthetic oligodeoxynucleotides 5'-ATCCTGGGAGTTGGaGATGTAGACTAGTTGGGTC-3' or 5'-ATGTCAGACTAGTgtGGTCATTGAAGG-3' encompassing nucleotides -112 to -79 or -97 to -70 of the human RAR β 2 promoter, respectively, and with the altered bases shown in lowercase letters. In a consensus CRE or TRE, these alterations have been shown to abolish the function of these elements (68, 78).

The adenovirus E2A promoter-containing CAT construct, p2CAT, and the E1A (Ad2) expression vectors pEIASV, pEIA 12S, and pEIA 13S, have been previously described (79, 80).

Plasmids used for the generation of 32 P-labeled riboprobes contained murine RAR α -, β -, and γ -specific cDNA subfragments (a 129-bp *Sst*I-*Pst*I fragment, a 116-bp *Eco*RI-*Pst*I fragment, and a 202-bp *Dde*I-*Apal* fragment, respectively) and a 58-bp *Hae*III fragment obtained from murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA subcloned in the pBluescript vector SK⁻ (Stratagene, La Jolla, CA) (81).

RNA Isolation and RNase Protection Analyses

Total cellular RNA was isolated using the acid-phenol single step method (82).

Complementary 32 P-labeled riboprobes directed against part of the C and D regions (RAR α), the F region (RAR β), and part of the E and total F regions (RAR γ) were transcribed using T3 or T7 RNA polymerases. Probes (10^4 - 10^5 cpm) were simultaneously hybridized to total RNA (15 μ g) overnight at 45 C in a buffer containing 80% (vol/vol) formamide, 40 mM piperazine-*N,N'*-bis-(2-ethanesulfonic acid) (pH 6.4), 0.4 M NaCl, and 1 mM EDTA. Subsequently, samples were digested with RNase-T1 for 30 min at 37 C, treated with 0.1 mg/ml proteinase-K and 0.5% sodium dodecyl sulfate, and extracted with phenol. After ethanol precipitation, the samples were run on a 6% polyacrylamide sequencing gel. The fixed gel was exposed at -80 C for 3 days to x-ray film using an intensifying screen. A

GAPDH complementary riboprobe was always included in the assay to correct for possible differences in the amount of RNA loaded in each lane (83). Transcript levels were quantified by using a Molecular Dynamics Phospho Imager with Image Quant software.

Transient Transfection Assays

END-2, END-2 E1A, P19 EC, HCT116, and 293 cells cultured on gelatin-coated dishes (diameter, 3.5 cm) were transfected by calcium phosphate coprecipitation (84) using 10 μ g CAT reporter plasmid DNA and varying amounts of E1A expression vectors in combination with 1 μ g SV2-LacZ as an internal control. After 16 h, fresh medium was added to the cells together with RA (final concentration, 10^{-6} M) when appropriate. Cells were harvested 24 h later and assayed for CAT activity, as previously described (85). The reaction products separated by TLC were quantified by using a Molecular Dynamics Phospho Imager (Eugene, OR) with Image Quant software. After normalizing transfection efficiency with the LacZ activity, CAT activity is expressed as the percentage conversion of the total amount of [14 C]chloramphenicol into its acetylated products. When using the p2CAT reporter plasmid, CAT activity is depicted as the percentage of that of the control, β -actin/CAT, expression of which is not modulated during differentiation. Routinely, transfection experiments were repeated at least three times.

Gel Retardation Assays

Nuclear protein extracts were prepared by the miniscale method, as described by Lee *et al.* (86). Protein concentrations were determined by the Bio-Rad protein assay according to the manufacturer's protocol.

The sequences of the double stranded oligonucleotides used in the assay were: CRE- β 2, TGAGTTGGTGATGTCA-GACTAG; and RARE- β 2, CCGGGTAGGGTTCACCGAA-AGTTCACCTCG.

The -180/-63 RAR β 2 promoter fragment used as a probe was generated by labeling the cohesive ends with [α - 32 P] deoxy-ATP using the Klenow fragment of DNA polymerase-I, followed by gel filtration using Sephadex G-50 spin columns (Pharmacia, Piscataway, NJ).

The electrophoretic mobility shift assay used is based on the procedures described by Fried and Crothers (87) with slight modifications. Nuclear extracts (4 μ g) were incubated in a reaction buffer (20 μ l) containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 75 mM KCl, 10% (vol/vol) glycerol, 1 mM dithiothreitol, 0.1 mg/ml poly-(dI-dC), 100 ng/ml pUC18 plasmid DNA, and 0.1 ng 32 P-labeled DNA fragment (SA, 1×10^6 / μ g) at room temperature for 20 min. An equal volume of loading buffer, containing 20% (vol/vol) Ficoll, was added, and the mixture was immediately loaded onto a 4% polyacrylamide gel and electrophoresed in $0.5 \times$ TBE (25 mM Tris, 25 mM boric acid, and 1 mM EDTA) for 4 h. The gel was fixed in 10% (vol/vol) methanol-10% (vol/vol) acetic acid, dried, and exposed to Kodak XAR film for 2 days.

Acknowledgments

We thank Siegfried de Laat and Christine Mummery for critically reading the manuscript, Maarten Mulder for making the END-2 E1A cell lines available, and A. H. Zantema and Rolf de Groot for providing the p2CAT construct and E1A expression vectors, respectively.

Received June 10, 1992. Revision received January 26, 1993. Accepted February 8, 1993.

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This work was supported by the Center for Developmental Biology (Utrecht, The Netherlands).

REFERENCES

1. Roberts AB, Sporn MB 1984 Cellular biology and biochemistry of the retinoids. In: Sporn MB, Robertson AB, Goodman DS (eds) *The Retinoids*. Academic Press, Orlando, vol 2:209-286
2. Durston AJ, Timmermans JPM, Hage WJ, Hendriks HFJ, De Vries NJ, Heideveld M, Nieuwkoop PD 1989 Retinoic acid causes an anterioposterior transformation in the developing central nervous system. *Nature* 340:140-144
3. Eichele G 1989 Retinoids and vertebrate limb pattern formation. *Trends Genet* 5:246-251
4. Jones-Villeneuve EMV, McBurney MW, Rogers KA, Kalnins VI 1982 Retinoic acid induces embryonal carcinoma cells to differentiate cell into neurons and glial cells. *J Cell Biol* 94:253-262
5. Edwards MKS, McBurney MW 1983 The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev Biol* 98:187-191
6. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* 240:889-895
7. Green S, Chambon P 1988 Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet* 4:309-314
8. Beato M 1989 Gene regulation by steroid hormones. *Cell* 56:335-344
9. Giguere V, Ong ES, Segui P, Evans RM 1987 Identification of a receptor for the morphogen retinoic acid. *Nature* 330:624-629
10. Petkovich M, Brand NJ, Krust A, Chambon P 1987 A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330:444-450
11. Benbrook D, Lernhardt E, Pfahl M 1988 A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature* 333:669-672
12. Brand N, Petkovich M, Krust A, Chambon P, De The H, Marchio A, Tiollais P, Dejean A 1988 Identification of a second human retinoic acid receptor. *Nature* 332:850-853
13. Krust A, Kastner P, Petkovich M, Zelent A, Chambon P 1989 A third retinoic acid receptor, hRAR- γ . *Proc Natl Acad Sci USA* 86:5310-5314
14. Zelent A, Krust A, Petkovich M, Kuster P, Chambon P 1989 Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. *Nature* 339:714-717
15. Hamada K, Gleason S, Levi BZ, Hirschfeld S, Appella E, Ozato K 1989 H-2 RIIIP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. *Proc Natl Acad Sci USA* 86:8289-8293
16. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM 1990 Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345:224-229
17. Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen J-Y, Staub A, Garnier J-M, Mader S, Chambon P 1992 Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 68:377-395
18. Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C 1992 9-*Cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68:397-406
19. Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huseilton C, Allenby G, Speck J, Kratzelstein Cl, Rosenberger M, Lovey A, Grippo JF 1992 9-*Cis* retinoic acid

- stereoisomer binds and activates the nuclear receptor RXR α . *Nature* 355:359–361
20. Doll P, Ruberte E, Kastner P, Petkovich M, Stoner CM, Gudas L, Chambon P 1989 Differential expression of genes encoding α , β and γ retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature* 342:702–705
21. Dolle P, Ruberte E, Leroy P, Morris-Kay G, Chambon P 1990 Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* 110:1133–1151
22. Ruberte E, Doll P, Krust A, Zelent A, Morriss-Kay G, Chambon P 1990 Spacial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. *Development* 108:213–222
23. Ruberte E, Dolle P, Chambon P, Morris-Kay G 1991 Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* 111:45–60
24. Kastner P, Krust A, Mendelsohn C, Garnier J-M, Zelent A, Leroy P, Staub A, Chambon P 1990 Murine isoforms of retinoic acid receptor γ with specific patterns of expression. *Proc Natl Acad Sci USA* 87:2700–2704
25. Leroy P, Krust A, Zelent A, Mendelsohn C, Garnier J-M, Kastner P, Dierich A, Chambon P 1991 Multiple isoforms of the mouse retinoic acid receptor α are generated by alternative splicing and differential induction by retinoic acid. *EMBO J* 10:59–69
26. Zelent A, Mendelsohn C, Kastner P, Krust A, Garnier J-M, Ruffenbach F, Leroy P, Chambon P 1991 Differentially expressed isoforms of the mouse retinoic acid receptor β are generated by usage of two promoters and alternative splicing. *EMBO J* 10:71–81
27. Hu L, Gudas LJ 1990 Cyclic AMP analogs and retinoic acid influence the expression of retinoic acid receptor α , β and γ mRNAs in F9 teratocarcinoma cells. *Mol Cell Biol* 10:391–396
28. Krut FAE, Van den Brink CE, Defize LHK, Donath M-J, Kastner P, Kruijer W, Chambon P, Van der Saag PT 1991 Transcriptional regulation of retinoic acid receptor β in retinoic acid-sensitive and -resistant P19 embryocarcinoma cells. *Mech Dev* 33:171–178
29. de The H, Vivanco-Ruiz MDM, Tiollais P, Stunnenberg H, Dejean A 1990 Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature* 343:177–180
30. Sucov HM, Murakami KK, Evans RM 1990 Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene. *Proc Natl Acad Sci USA* 87:5392–5396
31. Wang S-Y, LaRosa GJ, Gudas LJ 1985 Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyryl cyclic AMP in cultured mouse teratocarcinoma cells. *Dev Biol* 107:75–86
32. Vasios G, Mader S, Gold JD, Leid M, Lutz Y, Gaub M-P, Chambon P, Gudas L 1991 The late retinoic acid induction of laminin B1 gene transcription involves RAR binding to the responsive element. *EMBO J* 10:1149–1158
33. Glass CK, Devary OV, Rosenfeld MG 1990 Multiple cell type-specific proteins differentially regulate target sequence recognition by the α retinoic acid receptor. *Cell* 63:729–738
34. Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Näär AM, Kim SY, Boutin J-M, Glass CK, Rosenfeld MG 1991 RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 67:1251–1266
35. Kiewer SA, Umesono K, Mangelsdorf DJ, Evans RM 1992 Retinoid $\times$ receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D $_3$ signalling. *Nature* 355:446–449
36. Zhang X-K, Hoffmann B, Tran PB-V, Graupner G, Pfahl M 1992 Retinoid $\times$ receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* 355:441–446
37. Berk AJ 1986 Adenovirus promoters and E1A transactivation. *Annu Rev Genet* 20:45–79
38. Flint J, Schenk T 1989 Adenovirus E1a protein paradigm viral transactivator. *Annu Rev Genet* 23:141–161
39. Houweling A, Van der Elsen PJ, Van der Eb AJ 1980 Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology* 105:537–550
40. Ruley HE 1983 Adenovirus early region 1A enables viral and cellular genes to transform primary cells in culture. *Nature* 304:602–606
41. Land H, Parada LF, Weinberg RA 1983 Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304:596–602
42. Jones N, Shenk T 1979 An adenovirus type 5 early gene function regulates expression of other early viral genes. *Proc Natl Acad Sci USA* 76:3665–3669
43. Green MR, Treisman R, Maniatis T 1983 Transcriptional activation of cloned human β -globin genes by viral immediate-early gene products. *Cell* 35:137–148
44. Borrelli E, Hen R, Chambon P 1984 Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature* 312:608–612
45. Velich A, Ziff EB 1989 The adenovirus-5 12S E1a protein, but not the 13S induces expression of the endoA differentiation marker in F9 cells. *Oncogene* 4:707–713
46. Moran E, Mathews MB 1987 Multiple functional domains in the adenovirus E1A gene. *Cell* 48:177–178
47. Ferguson B, Krippl B, Andrisani O, Jones N, Westphal H, Rosenberg M 1985 E1A 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind to DNA. *Mol Cell Biol* 5:2653–2661
48. Chatterjee PK, Bruner M, Flint SJ, Harter ML 1988 DNA-binding properties of an adenovirus 289R E1A protein. *EMBO J* 7:835–841
49. Nevins JR 1981 Mechanisms of activation of early viral transcription by the adenovirus E1A gene product. *Cell* 26:213–220
50. Wu L, Rosser DSE, Schmidt MC, Berk A 1987 A TATA box implicated in E1A transcriptional activation of a simple adenovirus-2 promoter. *Nature* 326:512–515
51. Simon MC, Fisch TM, Benecke BJ, Nevins JR, Heintz N 1988 Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A regulation. *Cell* 52:723–729
52. Yee S-P, Branton PE 1985 Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. *Virology* 147:142–153
53. Harlow E, Whyte P, Franza BR, Schley C 1986 Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol Cell Biol* 6:1579–1589
54. Imperiale MJ, Kao H-T, Feldman LT, Nevins JR, Strickland S 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
55. La Thangue NB, Rigby PWJ 1987 An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. *Cell* 49:507–513
56. Hen R, Borrelli E, Fromental C, Sassone-Corsi P, Chambon P 1986 A mutated polyoma virus enhancer which is active in undifferentiated embryonal carcinoma cells is not repressed by adenovirus-2 E1A products. *Nature* 321:249–251
57. Linney E, Donerly S 1983 DNA fragments from PyEC mutants increase expression of heterologous genes in transfected F9 cells. *Cell* 35:693–699
58. Herbolme P, Bourachot B, Yaniv M 1984 Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* 39:653–662

59. Gorman CM, Rigby PWJ, Lane DP 1985 Negative regulation of viral enhancers in undifferentiated embryonic stem cells. *Cell* 42:519–526
60. Suemori H, Hashimoto S, Nakatsuji N 1988 Presence of the adenoviral E1A-like activity in preimplantation stage mouse embryos. *Mol Cell Biol* 8:3553–3555
61. Dooley TP, Miranda M, Jones NC, Melvin L 1989 Transactivation of the adenovirus E1a promoter in the absence of adenovirus E1A protein is restricted to mouse oocytes and preimplantation embryos. *Development* 107:945–956
62. Mummery CL, Feijen A, Van der Saag PT, Van den Brink CE, De Laat SW 1985 Clonal variants of differentiated P19 embryonal carcinoma cells exhibit epidermal growth factor receptor kinase activity. *Dev Biol* 109:402–410
63. de Groot RP, Foulkes N, Mulder M, Kruijer W, Sassone-Corsi P 1991 Positive regulation of *jun/AP-1* by E1A. *Mol Cell Biol* 11:192–201
64. Shen S-B, Kruyt FAE, Den Hertog J, Van der Saag PT, Kruijer W 1991 Mouse and human retinoic acid receptor β 2 promoters: sequence comparison and localization of retinoic acid responsiveness. *DNA Sequence* 2:111–119
65. Kruyt FAE, Folkers G, van den Brink CE, van der Saag PT 1992 A cyclic AMP response element is involved in retinoic acid-dependent RAR β 2 promoter activation. *Nucleic Acids Res* 20:6393–6399
66. Ziff EB 1990 Transcription factors: a new family gathers at the cAMP response site. *Trends Genet* 6:69–72
67. Liu F, Green MR 1990 A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. *Cell* 61:1217–1224
68. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herlich P, Karin M 1987 Phorbol ester inducible genes contain a common cis element recognized by a TPA modulated transacting factor. *Cell* 49:726–739
69. Lee W, Mitchell P, Tjian R 1987 Purified factor AP1 interacts with TPA inducible enhancer element. *Cell* 49:741–752
70. Murray EJ, Stott D, Rigby PWJ 1991 Sequences and factors required for the F9 embryonal carcinoma stem cell E1a-like activity. *Mol Cell Biol* 11:5534–5540
71. Graham FL, Smiley J, Russell WC, Nairu R 1977 Characteristics of a human cell line transformed by DNA from human adenovirus 5. *J Gen Virol* 36:59–72
72. Hoffmann B, Lehmann JM, Zhang X-K, Hermann T, Husmann M, Graubner G, Pfahl M 1990 A retinoic acid receptor-specific element controls the retinoic acid receptor- β promoter. *Mol Endocrinol* 4:1727–1736
73. Vivanco Ruiz MDM, Bugge TH, Hirschmann P, Stunnenberg HG 1991 Functional characterization of a natural retinoic acid responsive element. *EMBO J* 10:3829–3838
74. Spergel JM, Chen-Kiang S 1991 Interleukin-6 enhances a cellular activity that functionally substitutes for E1A-protein in transactivation. *Proc Natl Acad Sci USA* 88:6472–6476
75. Schöler HR, Ciesiolka T, Gruss P 1991 A nexus between oct-4 and E1A: implications for gene regulation in embryonic stem cells. *Cell* 66:291–304
76. Horikoshi N, Maguire K, Kralli A, Maldonado E, Reinberg D, Weinmann R 1991 Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. *Proc Natl Acad Sci USA* 88:5124–5128
77. Lee WS, Kao C, Bryant GO, Liu X, Berk AJ 1991 Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor IID. *Cell* 67:365–376
78. Foulkes NS, Borrelli E, Sassone-Corsi P 1991 CREM gene- use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* 64:739–749
79. Weeks DL, Jones NC 1983 E1A control of gene expression is mediated by sequences 5' to the transcriptional starts of the early viral genes. *Mol Cell Biol* 3:1222–1234
80. Leff T, Elkaim R, Goding C, Jalilnot P, Sassone-Corsi P, Perricaudet M, Kedinger C, Chambon P 1984 Individual products of the adenovirus 12S and 13S E1a mRNAs stimulate viral E1a and E1b expression at the transcriptional level. *Proc Natl Acad Sci USA* 81:4381–4385
81. Jonk LJC, De Jonge MEJ, Kruyt FAE, Mummery CL, Van der Saag PT, Kruijer W 1992 Aggregation and cell cycle dependent retinoic acid receptor mRNA expression in P19 embryonal carcinoma cells. *Mech Dev* 36:165–172
82. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
83. Fort PH, Marty L, Piechaczyk M, ElSabrouty S, Dnai Ch Jeanteur Ph Blanchard JM 1985 Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res* 13:1431–1422
84. Banerji J, Rusconi S, Schaffner W 1981 Expression of a β -globin gene is enhanced by remote SV40 DNA sequences. *Cell* 27:299–308
85. Gorman CM, Moffat LF, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Mol Cell Biol* 2:1044–1051
86. Lee KAW, Bindereif AS, Green MR 1988 A small scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gen Anal Technol* 5:22–31
87. Fried M, Crothers DM 1981 Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res* 9:6505–6525