A cis-acting element 7 bp upstream of the ESF-1-binding motif is involved in E1A 13S autoregulation of the adenovirus 12 TS2 promoter

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Introduction

The adenovirus (Ad) E1A oncogene is located in the leftmost 5% of the adenovirus genome and is transcribed immediately after infection (Berk et al., 1979). It codes for two predominant proteins, 266R and 235R, translated from the 13S and 12S mRNA. Both proteins fulfil multiple regulatory functions, including transcriptional activation or inhibition of viral (Berk et al., 1979) and cellular genes (Brockmann et al., 1995; De Groot et al., 1991). Moreover, they are essential for the immortalization of primary rodent cells (Lynch & Trainer, 1989) and for complete cell transformation in co-operation with functions of other oncogenes like E1B and ras (Branton et al., 1985).

In host cells, expression of E1A is a strictly controlled process and previous reports have shown that the E1A protein(s) itself take(s) part in this control (Cogan et al., 1992; Larsen & Tibbetts, 1987). Evidence from transient expression assays suggests that Ad12 E1A transcription is positively autoregulated by its largest gene product, the 13S protein (Kirch et al., 1993; Yamazaki et al., 1992), but is not affected through the protein translated from the 12S mRNA (Yamazaki et al., 1992).

The work of several researchers has indicated that the 13S E1A protein regulates transcription by modifying the activity of multiple cellular promoter binding factors (e.g. E2F, E4F, ATF, AP1 and TFIID) (Jones, 1992). For example, the large E1A protein associates with different members of the ATF-CREB transcription factor family (ATF-2, ATFa proteins) (Chatton et al., 1993). ATF-2 appears to mediate the E1A response by bringing E1A in close proximity to the basal transcription factors.
transcriptional machinery (Lewin, 1990). Additionally, E1A-dependent activation of certain promoters results from direct interaction with the TATA-box-binding protein, TBP (Boyer & Berk, 1993). Transactivation mediated by the 13S protein as well as the 12S protein was also due to release of the transcription factor E2F from pre-existing inactive complexes containing the p105 \( ^{68} \) tumour suppressor protein or the related p107 or p130 pocket proteins (Bagchi et al., 1990). In the case of indirect functions ascribed to the E1A proteins, E1A expression is correlated with increased phosphorylation and activity of E2F (Bagchi et al., 1989), E4F (Raychaudhuri et al., 1989) and TFIIC (Hoeffler et al., 1988).

In contrast to Ad2 and Ad5 (Berk, 1986), the mechanism of autoactivation of Ad12 E1A is not yet understood. Because of significant differences in the promoter regions in both E1A genes it is reasonable to assume that the mechanism of autoactivation might be different. For example, multiple cellular transcription factor binding sites identified so far in the Ad5/Ad2 promoter could not be detected in the E1A transcriptional control region of Ad12. Furthermore, Ad12 possesses two functional transcriptional start sites located at nt 306 (TS1) and 445 (TS2). Both initiation sites are active at an early stage after infection with virus (Saito et al., 1981; Sawada & Fujinaga, 1980), resulting in a long and a short 13S- and 12S-primary transcript (Fujinaga et al., 1984; Nakamichi et al., 1987; Shibata et al., 1989).

One way to understand the mechanism of autoregulation of the Ad12 E1A gene might be to identify DNA-binding sites for trans-acting factors, the activity of which is apparently regulated by E1A. Thus, for Ad12 it was shown by in vitro transcription assays that the promoters of both TS1 and TS2 are individually stimulated by cellular factors (Shibata et al., 1989; Nakamichi et al., 1987). Previous reports have indicated that the binding of nuclear factor-I (NF-I) to a region located near the left end of the Ad12 genome is involved in stimulation of TS1 (Koikeda et al., 1990; Shibata et al., 1989) and that the DNA-binding activity of NF-I might be inhibited by the E1A protein (Koikeda et al., 1990; Shibata-Sakurai et al., 1991). In addition, the E1A-stimulating factor 1 (ESF-1) was found to activate basal transcription from TS2 by interacting with a palindromic DNA sequence immediately upstream of the TATA box (Shibata-Sakurai et al., 1991, 1993). In in vitro assays using purified E1A 13S protein, TBP was identified as a target for E1A-mediated activation of the proximal TS2 promoter (Kawamura et al., 1994).

Our previous studies have demonstrated that the stimulation of TS1 by co-operative activation of an E2F motif and a potential ATF motif is responsible for E1A-independent and 13S-activated expression of the E1A gene (Kirch et al., 1993). To investigate the role of TS2 in E1A autoregulation, we examined the promoter region upstream of TS2 for E1A responsive elements in transient expression assays. We identified a cis-acting DNA-binding motif which resembles an E2F transcription factor binding site, and which is important for E1A-mediated activity of TS2. In addition, our data indicate a synergism between both promoter regions with respect to the 13S protein-induced autoactivation of the Ad12 E1A gene, which is mediated by the E2F motifs in both promoters.

### Methods

**Cell lines and growth conditions.** Cell culture media were obtained from Gibco BRL. The human embryonic kidney cell line HEK 12 (kindly provided by P. Gallimore, Institute of Cancer Studies, University of Birmingham, UK), Hela and A549 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with heat-inactivated 10% foetal calf serum. HEK cells constitutively express E1 products of Ad12. The Raji human Burkitt’s lymphoma cell line (ATCC) was cultured in RPMI 1640 medium supplemented with heat-inactivated 10% foetal calf serum.

**Synthetic oligonucleotides used in gel-shift assays (GSA).** The oligonucleotides were synthesized and electrophoretically purified as described in Kirch et al. (1993). The following oligonucleotides (sequences of the coding strand only are shown) were used as probes: oligo-E-Box (nt 388–408 of the Ad12 E1A region), 5’ - (GGG)ATTGCATCTGACGCTATGTG 3’, containing the ESF-1-binding site (factor binding sites are shown in italics, the E-Box sequence is underlined, and parentheses enclose G’s that were added 5’ for radioactive end-labelling); oligo-E2FIIL, 5’ - (GGG)CCCTCTAGGCGCCTTTATT 3’, corresponding to the region between positions 370–390 including the E2F-like sequence 7 bp upstream of the palindromic E-Box; and oligo-TATA (nt 407–425), 5’ - GTTTGGAGATTTAATCCGG 3’, containing the proximal TATA-box of the E1A gene described in Kawamura et al. (1994). For use as probes in GSA the oligonucleotides were 3’ end labelled with [\( ^{32} \)P]dCTP. The following oligonucleotides were used only as competitors: oligo-RND, 5’ - (GGG)CGCAGATGATAGAGATAGAGAGAGAAAAA 3’, a randomly chosen sequence from the E1B gene of Ad12 (nt 2091–2119); E2F(a), containing the E2F sequence of Ad12 upstream of TS1 (described in Kirch et al., 1993); oligo 71/50, 5’ - (GGG)TAGTTTCCGGGGTTTAATTGA 3’, containing the first E2F motif of Ad5 E2 promoter; oligo 62/60, 5’ - (GGG)TAGTTTTCCGGGGTTTAATTGA 3’, with point mutations in positions 60–62 in the first E2F motif of Ad5 E2 (shown in lower-case letters); oligo E-Box-mut, 5’ - ATTGTCTGTGCGCTGATCGTTTG 3’, modified by two point mutations in the proximal ESF-1-binding site (M2) described in Shibata-Sakurai et al. (1993); and oligo TATA-mut, 5’ - TGGGATCGAATTCCGCGCGTATGTTTTCCGGGGTTTAATTGA 3’ (mutated proximal TATA-box of Ad12 E1A).

**Antibodies used in GSA and Western blotting.** Monoclonal mouse antiserum directed against E2F-5 (sc-968), p107(sc-2500X) and Ku(sc-466) together with the polyclonal rabbit antibodies E2F-1 (sc-193X), E2F-2 (sc-632X), E2F-4 (sc-512X), p130 (sc-317X) and Rb (sc-50X) were obtained from Santa Cruz, Biotechnology and used in GSA. For Western blot analysis a monoclonal anti-Myc antibody (hybridoma supernatant) was kindly provided by L. Klein-Hitpaß, Institute of Cell Biology, University of Essen, Germany.

**Preparation of nuclear extracts.** Extracts were prepared from cells grown to 70–80% confluence (1·5–2 x 10^6 cells/ml). Cell pellets were washed with PBS and stored at −20 °C overnight. After thawing of frozen pellets on ice cells were lysed with 10 mM HEPES, 0·5 mM spermidine, 0·15 mM spermine, 1 mM EDTA, 0·25 mM EGTA, 0·5 M sucrose, 50 mM NaCl, 1 mM DTT, 2 µg/ml aprotinin, 20 µg PMSF/ml, pH 7·9 (buffer A) for 15 min on ice. The degree of cell lysis was controlled by phase-contrast microscopy. Thereafter, nuclei were washed
three times with buffer B (buffer A plus 0.35 mM sucrose). Histone-free nuclear extracts were obtained by careful treatment (slow rocking or stirring) of the nuclei in 420 mM NaCl in buffer B for 30 min on ice. The extracts were centrifuged at 100,000 g for 30 min at 4 °C and the supernatants were immediately frozen in liquid nitrogen and stored at −80 °C. The protein concentration of the nuclear extracts was determined using the Bio-Rad Protein Assay.

**Gel-shift assay.** This was done as described in Barrett et al. (1987) with minor modifications: the end-labelled oligonucleotide (0–1–0.5 ng) was incubated with nuclear proteins (1–2 µg) and unspliced DNA [1000-fold molar excess of poly(dA)–(dT)] for 30 min in a 15–20 µl binding-reaction mixture consisting of 20 mM HEPES and 150 mM NaCl, pH 7.4. In a competition assay, competitor oligonucleotide was added to the binding-reaction mixture at a 50–to 200-fold molar excess relative to the radiolabelled probe. Antibody perturbation experiments of oligoDNA–protein complexes were done by preincubating the nuclear extract with 1 µg of the appropriate antibody overnight at 4 °C prior to the binding-reaction. The mixture was then loaded onto a gel containing 6% polyacrylamide and electrophoresed for 2–3 h at 200 V. Gels were fixed with 14% acetic acid, dried and exposed to autoradiography films.

**CAT constructs.** Promoter elements of interest (shown in Figs 2A and 4A) were amplified by PCR. For generation of the E1A promoter construct WT-199–341, the forward primer 5’ (CTAAAGCCTT-TGGGCGTTGTTTGGCAGAAATTTTG). The proximal TATA box upstream of TS2 (shown in parentheses) encloses the artificial restriction site added 5’ of the E1A promoter sequence.

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![Fig. 1. Schematic representation of the adenovirus 12 E1A promoter (nt 1–456). The distal promoter region upstream of TS1 and the proximal region upstream of TS2 are indicated. DNA-binding motifs of nuclear factors and TATA boxes are shown. The terminal binding protein (TP) covalently binds the 5’ ends of DNA.](image-url)
resuspended in cytomix (Van den Hoff et al., 1992); 225 µl of cells and 25 µl of DNA were mixed and pipetted carefully into the bottom of a new 0.4 cm cuvette (Bio-Rad). After incubation on ice for 20 min electroporation was performed by increasing the voltage to 250–300 V (optimized for each cell line) and setting the capacitance to 960 µF. Cells were immediately put back on ice for 10 min, resuspended in 8 ml of DMEM supplemented with 10% foetal calf serum, plated into Petri dishes and incubated for 48 h at 37 °C. CAT assays were carried out using the diffusion technique described by Neumann et al. (1987). Cells were washed twice with PBS, scraped from the dishes, harvested and resuspended in 50 µl 0.25 M Tris, pH 7.8. Cells were lysed by freezing/thawing, the cytosolic extract cleared by centrifugation at 12,000 g and the protein concentration determined (Bio-Rad protein assay). Cell-extract protein (50 µg) was incubated in 0.25 M Tris, 1.6 mM chloramphenicol and 0.49 mM [3H]acetyl-CoA (0.5 µCi) for 30 min at 37 °C. After adding 4 ml scintillation fluid (Econofluor NET-290L; NEN DuPont) to the mix and further incubation at 37 °C radioactivity was measured by liquid scintillation counting over a 5 h period. To ensure 13S cDNA expression after transfection, cells were transfected with the pCS2­MT-6myc-13S and Western blotting.

PCR amplification of the 13S cDNA of Ad12-E1A was done using the forward primer 5’-(GGCGGAAGCTT)ATGAGAACTGAAATGACTC (nt 502–520; the artificial HindIII site is enclosed by parentheses) and the backward primer 5’-(GGCGGAAGCTT)AGGTGCTCAAAGTCCATA 3’ corresponding to the region between nt 1374–1392 of Ad12 E1A. The PCR product was cloned into the multiple cloning site of the pCS2­MT-6myc plasmid using the HindIII restriction site of the primers. Nuclear extract of the cell lines transfected with the pCS2+MT-6myc-13S construct was separated by SDS–PAGE and transferred to nitrocellulose membranes (Hybond ECL, Amersham). The blot was blocked with 10% nonfat dry milk powder (NFMP) in PBS–0.1% Tween 20 (PBS-T) and incubated with the primary antibody (1:4) in PBS-T with 5–10% NFMP for 1 h at room temperature. After four washing steps with PBS-T, the antigen was detected by using horseradish peroxidase (HRP)-coupled secondary mouse antibody against Myc, dilution 1:5000 in 0.5% NFMP and the ECL detection system (Amersham).

Results

The E2FII, the E-Box and the flanking TATA box sequence are important for TS2 promoter activity in the absence of E1A

Computer-supported sequence analysis of the TS2 promoter region revealed a cis-acting DNA-binding motif, which might resemble an E2F transcription factor binding site when read 3'→5', located immediately upstream of a palindromic E-Box and its 3'-flanking TATA element (Fig. 1). Based on this sequence similarity it is referred to as E2FII in the Ad12 E1A promoter. To analyse the participation of these elements in the activation of E1A gene expression in the absence of E1A products we performed transient expression assays in A549, Raji and HeLa cells. The lung carcinoma cell line A549 and the lymphoblast-like Raji cells, established from human Burkitt's lymphoma, were both used as they represent human cells permissive for adenovirus infection (Lavery et al., 1987). For these experiments a series of constructs (shown in Fig. 2A) containing either each of the binding sites (WT-TS2-CAT) or the E-Box plus the TATA box (-E2FII/TATA-CAT) as well as the
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Fig. 2. Role of the E2FII-, E-Box- or TATA box elements in E1A-independent and E1A-mediated transcriptional activation. (A) Schematic diagram of CAT reporter constructs. The different constructs contain the nuclear factor binding sites E2FII, E-Box and the TATA box located in the TS2 promoter region as well as TS2. The mutations in the E-Box and the TATA box are indicated as crossed boxes. TS2 is marked with an arrow. All constructs were fused to the CAT reporter gene of the pCAT-Basic vector. (B) The nucleotide sequences of the E-Box and TATA box oligonucleotides and their mutant derivatives (in lower-case letters, indicated by arrows) are shown (the palindromic E-Box motif and the TATA box are underlined; the ESF-1 binding site is shown in italics). (C) A gel-shift experiment was done with either the E-Box- (lanes 1–5) or TATA oligonucleotide (lanes 6–9) as a probe using Raji cell nuclear extract. Unlabelled competitor oligonucleotides containing the E-Box (lane 3) or the TATA box (lanes 4 and 8) as well as the mutated E-Box sequence (M2) described in Shibata-Sakurai et al. (1993) or the mutated TATA box (-E2FII/-E-BOX/TS2-CAT) (lanes 2 and 7), respectively, were added at a 200-fold molar excess of probe. A randomly chosen Ad12 E1B sequence (RND) was used as a control (lanes 5 and 9). The positions of the complexes 'a', 'b', 'c' and 'd' as well as of the free probe are indicated. A non-specific DNA–protein complex is marked (●). The complexes were separated by PAGE (6% polyacrylamide). (D) Relative CAT activity expressed from the different Ad12 TS2 promoter constructs was examined in A549, Raji and HeLa cells. Diffusion of [3H]acetylated chloramphenicol into scintillation fluid was determined after 5 h by scintillation counting. The promoterless construct pCAT-Basic was used as control and is its activity is defined as 1. Standard deviations were as indicated. (E) Relative CAT activities of the promoter constructs were examined in A549, Raji and HeLa cells co-transfected with the pRc/RSV-1.3S expression vector containing the 1.3S cDNA of Ad12 E1A, as well as in transformed HEK12 (Ad12 E1A) cells. The activities shown are average values from at least three independent experiments.
TATA box alone (-E2FI/-E-BOX/TS2-CAT) were produced. Additionally, the E-Box motif within the construct containing all binding sites (E-BOXmut/TS2-CAT) as well as the TATA sequence of the E-Box motif plus TATA motif (-E2FI/TATAmut/TS2-CAT) were destroyed by creating point mutations (Fig. 2B).

To examine whether the TATA or E-Box mutations of the CAT constructs correlate with loss of the ability to compete for factor binding to the corresponding motif, oligonucleotides containing the mutated TATA or E-Box sequences (designated M2; as described in Shibata-Sakurai et al., 1993) were tested in gel-shift assays as competitors. We applied nuclear extract prepared from Raji cells and radiolabelled oligonucleotides containing either the TATA box or the E-Box motif as probes. For the M2 oligonucleotide it was previously shown (Shibata-Sakurai et al., 1993) that mutations at the proximal site of the ESF-I footprint (5′ TGTCA 3′; Fig. 2B) deprived the oligonucleotide of the ability to compete for ESF-I binding. As shown in Fig. 2(C), for both binding sites several DNA–protein complexes could be detected (lanes 1 and 6); major complexes were labelled ‘a’, ‘b’ or ‘d’, and two additional complexes were labelled ‘c’. All three E-Box complexes (lane 1) as well as the complexes formed with the TATA box oligonucleotide (lane 6) were significantly reduced by adding unlabelled E-Box (Fig. 2C, lane 3) or TATA box oligonucleotide (lane 7), respectively, at a 200-fold molar excess. In contrast, complex formation was not affected by using the same amount of an unspecific control oligonucleotide, RND, as a competitor (randomly chosen E1B sequence from nt 2091–2119; Fig. 2C, lanes 5 and 9), except for the complex indicated by (●) which is therefore expected to be non-specific. Additionally, the E-Box complex ‘b’ was not affected by using the TATA box oligonucleotide as a competitor, whereas both other complexes were significantly reduced (lane 4). On the other hand, both the E-Box and the M2 oligonucleotides efficiently compete all TATA box complexes (data not shown), which indicates binding of an E-Box-specific factor for complex ‘b’ and a cross-affinity between the E-Box- and the TATA box-binding proteins of all other complexes.

Of note, neither the TATA box complexes ‘a’, ‘c’ and ‘d’ (lane 4) nor the apparently ESF-I-specific E-Box complex ‘b’ (lane 2) were affected by an excess amount of the corresponding mutated sequence, indicating the specificity of the mutations tested in CAT assays (Fig. 2C).

To test the promoter activity of the TS2-binding elements in transient expression assays, various constructs which all contained the Ad12 E1A promoter TS2 (Fig. 2A) were fused to the CAT reporter gene using the pCAT-Basic vector and introduced into growing A549, Raji and HeLa cells by electroporation. CAT assays were performed 48 h later, and CAT activity determined after 5 h was compared with that of the control vector pCAT-Basic, which was set to 1. The WT-TS2-CAT construct containing the E2FI, the E-Box as well as the TATA element caused significant CAT activation, ranging from about 8-fold (HeLa and Raji cells) to 10-fold (A549 cells) in the absence of E1A (Fig. 2D), indicating that the TS2 promoter in all these cell lines is active. This complete promoter activity was reduced by half when the E-Box motif (E-BOXmut/TS2-CAT) was mutated. On the other hand, a comparable (4- to 5-fold) decrease in CAT expression was observed for the construct containing the E-Box in front of the TATA box (-E2FI/TS2-CAT). Transcriptional activation decreased to only 2- to 3-fold for the E-Box construct that contains the mutated TATA box (-E2FI/TATAmut/TS2-CAT) as well as the TATA box alone (-E2FI/-E-BOX/TS2-CAT) in all transfected cell lines. From these results we conclude that the E2FI motif as well as the E-Box resulted in an additive effect on CAT expression with regard to the TATA construct, indicating the involvement of all three DNA-binding sites in E1A-independent gene expression from the TS2 promoter region. Additionally, it seems that the cellular proteins which bind to the E2FI motif as well as to the other elements are constitutively expressed in all three cell lines under our defined growth conditions. Surprisingly, promoter activity was not eliminated by incorporation of a mutated TATA sequence into the construct containing the E-Box (-E2FI/TATAmut/TS2-CAT). Compared with the TATA box (-E2FI/-E-BOX/TS2-CAT) the latter construct had the same transcriptional activity. This might indicate that the proximal TATA box is functionally substituted by the E-Box sequence.

**The E2FI-binding sequence is required for E1A 13S-stimulated TS2 promoter activity**

Our previous report had shown that the TS1 promoter of Ad12 was positively autoregulated by the E1A 13S protein (Kirch et al., 1993). To investigate whether the E1A TS2 promoter is also regulated by the 13S mRNA product, and to determine the elements required for promoter activity stimulated by this E1A protein, we carried out transient CAT expression assays. In this context it should be noted that the
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Fig. 4. Identification of an E2F-like binding activity for the E2FII sequence of the Ad12 TS2 promoter region. (A) Nuclear extract of Raji cells was examined for binding to 32P-labelled oligonucleotide probe E2FII in the presence of various competitors in a gel-shift assay. The following competitor DNAs were added at the indicated molar excess of probe: for self-competition the E2FII oligonucleotide containing the E2F-like motif of the TS2 promoter (lanes 2 and 3), the specific wild-type E2F DNA sites from Ad5 E2 promoter (E2F 71/50; lanes 4 and 5) and Ad12 TS1 promoter [E2F(a); lanes 6 and 7] and the mutated sequence 71/50 (E2F 62/60; lane 9), unable to compete for specific E2F complexes (La Thangue et al., 1990). As a control, a randomly chosen sequence from Ad12 E1B (RND) was used. The DNA–protein complexes ‘a’ and ‘d’ are marked. A non-specific DNA–protein complex is indicated (E). (B) For antibody perturbation experiments the E2FII probe was used with Raji nuclear extract. To the reaction mixture was added 1 µg of antibody directed against the various E2F factors E2F-1 (sc-193; lane 2), E2F-2 (sc-632; lane 3), E2F-4 (sc-512; lane 4) and E2F-5 (sc-968; lane 5) as well as against cellular pocket proteins p130 (sc-317; lane 7), p107 (sc-250; lane 6) and the control antibody Ku directed against the Ku protein (sc-466; lane 10). Extracts were separated by PAGE (6% polyacrylamide).

12S mRNA product of Ad12 was previously shown not to be involved in positive regulation of E1A gene expression (Yamazaki et al., 1992). A pRc/RSV-13S cDNA expression vector, encoding the 13S mRNA of the Ad12 E1A gene, was co-transfected into human A549, Raji and HeLa cells together with the TS2 promoter constructs (shown in Fig. 2A), as described above (E1A 13S expression by the co-transfected cells was tested beforehand by detection of the Myc epitope tags in the 5' position of 13S, described in Methods; data not shown). The same constructed CAT plasmids were used in transfection of the Ad12 E1-transformed HEK cells. CAT activity was compared with that of the pCAT-Basic vector after co-transfection with the 13S-expression vector (defined as 1). Fig. 2(E) shows that in co-transfection experiments the construct containing all three binding elements (WT-TS2-CAT) gave the highest level of CAT expression (compared with pCAT-Basic), approximately 19-fold in A549 cells, 18-fold in Raji and somewhat lower in HeLa and HEK 12 cells (about 12-fold). Altogether, comparing the complete TS2 promoter activity in the presence and absence of the 13S protein in HeLa, A549 and Raji cells, this E1A product caused a significant induction of gene expression. Between all three cell lines a comparable and only moderate decrease in 13S-mediated gene expression of the WT-TS2-CAT construct was ascertained for the plasmid E-BOXmut/TS2-CAT containing the mutated E-Box sequence, resulting in an almost 15-fold stimulation of the pCAT-Basic level in A549 and Raji cells, and about 9-fold in HeLa and Ad12 E1-transformed HEK cells. In comparison with the E1A-independent activity measured for this construct (carrying the E2FII motif and the TATA box), the 13S protein induced a 2- to 3-fold induction of reporter gene activity. In contrast, CAT expression detectable for the promoter construct -E2FII/TS2-CAT decreased to approximately 6-fold, which is only insignificantly higher than in the absence of the E1A product. Interestingly, independent of the cell line analysed, neither the TATA box nor the E-Box motif alone was significantly stimulated by the 13S protein. All these results suggest that the E1A TS2 promoter is positively autoregulated and that the E2FII region is necessary and sufficient for autoregulation.
Fig. 5. For legend see facing page.
The E1A TS2 promoter of Ad12 is bound by an E2F-5-like protein

To investigate which cellular proteins are involved in binding to the TS2 E2FII motif we performed gel-shift competition experiments using the E2FII oligonucleotide as the labelled probe and nuclear extract from HeLa, A549 and Raji cells grown to 70–80% confluence. Based on its sequence (Fig. 3), we postulated that the E2FII motif might be a possible binding site for E2F. Therefore, as competitor DNAs we used the E2F motif of Ad12 E1A [designated E2F(a); Kirch et al., 1993] and the first E2F site (71/50) from the Ad5 E2 promoter (Yee et al., 1989) as well as the mutated 71/50 sequence [designated 62/60; as described in La Thangue et al. (1990); the oligonucleotide 62/60 did not compete for specific E2F complexes]. All cell lines investigated form identical DNA–protein complexes (data not shown). The experiments shown were performed with Raji cell extract. For the E2FII oligonucleotide two complexes, ‘a’ and ‘d’ (Fig. 4A, lane 1) and a third complex indicated by ‘○’ (also competed by a randomly chosen E1B sequence and thus expected to be non-specific; RND, lane 8) could be detected. To our surprise, both complexes ‘a’ and ‘d’ were significantly reduced by a 50-fold molar excess of both oligonucleotides containing E2F-specific binding sites (lanes 4 and 6), and were nearly as effective as self-competition (lane 2). Both competitors completely abolished protein-binding at a 200-fold molar excess (lanes 5 and 7), whereas this was not affected by an excess of the competitor DNA 62/60, or the control oligonucleotide RND (lanes 8 and 9).

To directly demonstrate that an E2F-like protein binds to the E1A TS2 promoter DNA, an antibody perturbation experiment with antisera directed against various E2Fs and E2F-associated cellular proteins was carried out (Fig. 4B). A mouse monoclonal antibody recognizing the E2F-5 protein and a polyclonal serum directed against p130 were able to prevent the formation of complex ‘d’ (Fig. 4B, lanes 5 and 8). It is known that human E2F-5 preferentially interacts with p130 in GSA as well as under physiological conditions (Buck et al., 1995; Hijmans et al., 1995). Interestingly, the nuclear extracts used for antibody perturbation experiments were prepared from 70–80% confluent cells, and consistent with this E2F-5–p130 complexes were found predominantly in quiescent cells (Hijmans et al., 1995). To ensure the specificity of the E2F-5 antibody it was tested with probes containing specific factor-binding sites distinct from E2F in GSA like CREB- and NFξB-binding sites. As expected, no supershift or displacement were obtained in these experiments (data not shown). In addition, complex ‘d’ was also displaced by the monoclonal anti-p107 antibody (Fig. 4B, lane 7). Although neither the p130 nor the anti-p107 antibody affected the more rapidly migrating gel-retarded species, the anti-E2F-5 antiserum additionally abolished the DNA–protein complex ‘a’ (lane 5). In contrast, antisera directed against the other E2F family members, E2F-1, 2, 3 and 4 (lanes 2–4), or the nuclear pocket protein p105RB as well as the control antibody anti-Ku (lane 6 and 10), had no effect on complex formation. Thus, despite the fact that the binding sequence is not identical to the consensus E2F site, the protein present in the Raji nuclear extract which bound to the E2FII motif of the Ad12 E1A TS2 promoter region is immunologically related to E2F-5.

**The 13S protein is responsible for synergistic activation of E1A expression from TS1 and TS2**

Next, we examined the effect of the 13S protein of Ad12 on the E1A promoter region located between nt 199–456 (designated WT-TS1/+ WT-TS2-CAT), containing both TS1 and TS2. Fused to the CAT reporter gene, the plasmid was used in combination with the constructs WT-TS1-CAT (nt 199–TS1; Kirch et al., 1993) and WT-TS2-CAT (nt 349–TS2) in transient transfection experiments in A549 cells with and without the 13S protein (Fig. 5A). Since the CAT activity of both E1A promoters seems to be dependent on the E2F or E2F-like binding site in each promoter, two other typical E2F-motif-containing promoters, the EIIA-early WT CAT and the hamster pDHF-240 CAT, were compared in the absence and presence of the Ad12 13S protein. As shown in Fig. 5(B), E1A-independent CAT activity detected for the construct WT-TS1/+ WT-TS2-CAT was similar to the activity measured for each individual E1A promoter region as well as for the E2F-motif-containing Ad5 EIIA or DHFR promoters (about 10-fold compared with pCAT-Basic). Most interestingly, the expression of WT-TS1/+ WT-TS2-CAT was induced 6-fold compared with the E1A-independent trans-activation, whereas WT-TS1-CAT and WT-TS2-CAT show only a 2-fold activation. In comparison with the pCAT-Basic vector (13S-co-transfected) we measured a 30-fold stimulation for WT-TS1-CAT, and 15-fold for WT-TS2-CAT, resulting in a 70-fold activity of WT-TS1/+ WT-TS2-CAT. This result suggests a synergistic interaction between TS1 and TS2 in autoactivating

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**Fig. 5.** Synergistic activation of Ad12 E1A promoter regions TS1 and TS2 by the 13S protein correlates with functional E2F sites. (A) Schematic diagram of the Ad12 E1A promoter constructs WT-TS1-CAT, WT-TS2-CAT, WT-TS1/+WT-TS2-CAT (containing both E1A promoter regions) and the mutants TS1-E2Fmut/+WT-TS2-CAT and WT-TS1/+TS2-E2Fmut-CAT as well as the CAT constructs EIIA-early WT CAT containing the E2 promoter of Ad5 described in Manohar et al. (1990) and the hamster dihydrofolate reductase promoter pDHF-210 CAT (Blake & Azizkhan, 1989) used for transient expression assays. The mutations in the E2F sites are indicated as crossed boxes fused to the CAT reporter gene. Nuclear factor binding sites are shown as boxes and start sites of transcription are indicated by an arrow. (B, C) Relative CAT activity expressed from the promoter constructs in A549 cells co-transfected and not co-transfected with the pRC/RSV-13S vector expressing the 13S protein of Ad12. Promoter activity of the control plasmid pCAT-Basic was defined as 1.
the E1A gene. The Ad5 E1IA-early promoter, which possesses two E2F motifs, was induced about 5-fold by the 13S protein of Ad12, but compared with the entire Ad12 E1A promoter region (nt 199–456) full promoter activity was significantly lower (Fig. 5B). Moreover, the 13S-induced WT-TS2-CAT activity shows similarity to reporter gene expression of the DHFR promoter, consistent with the idea that both promoters might contain similar E2F sequences (reading 3′-to-5′; Fig. 3). The DHFR site possesses dyad symmetry and does include two potential overlapping E2F-binding sites. However, the binding of only one E2F protein was noted, at least in vitro (Blake & Azizkhan, 1989). All these results indicate that the TS1 as well as the TS2 promoter region is individually stimulated by the E1A gene product of Ad12, and that both promoter regions synergistically co-operate in E1A autoactivation.

The TS1/TS2 synergism is mediated by the E2F-binding sites in both E1A promoter regions

To determine the E1A promoter motifs necessary for the 13S-mediated synergistic activation, we constructed promoter mutants in which either of the E2F-binding sites was destroyed by introducing point mutations (Fig. 5A). For mutating the E2F motifs, we modified the nucleotides 5′ CGC 3′ of the E2F consensus sequence to 5′ ATA 3′, which was shown to correlate with the loss of competition for E2F-specific complexes (La Thangue et al., 1990; see also 62/60-oligo in Fig. 4A, lane 9). The construct TS1-E2Fmut/+ WT-TS2-CAT, containing the modified E2F site upstream of TS1, and construct WT-TS1/+ TS2-E2FIImut-CAT, with the mutated TS2 promoter site, were analysed in transient expression assays in A549 cells with and without the Ad12 13S protein (Fig. 5C). In the absence of E1A, CAT expression from the WT-TS1/+ WT-TS2-CAT wild-type reporter construct was induced about 11-fold compared with the pCAT-Basic vector (defined as 1). CAT expression decreased to 30% for the construct that contains the nonfunctional E2F site of the TS1 promoter region (TS1-E2Fmut/+ WT-TS2-CAT) and to 50% for the WT-TS1/+ TS2-E2FIImut-CAT promoter mutant, indicating an additive effect of both E2F-binding sites on E1A-independent gene expression. Cotransfection of the WT-TS1/+ WT-TS2-CAT construct with the 13S expression vector results in about 90-fold induction of E2F-specific activity (8-fold induction of reporter activity by the E1A protein, Fig. 5C). This 13S-mediated effect was reduced to only 10-fold if the TS1 E2F-motif was mutated and to 18-fold for the construct containing the mutated E2FII site. These results clearly show that the level of 13S-induced promoter activity detected for the WT-TS1/+ WT-TS2-CAT construct depends on the functional E2F sites in both E1A promoter regions and indicates that the synergistic effect observed between the TS1 and the TS2 promoters (described in Fig. 5B) is mediated by both E2F motifs.

Discussion

The data presented in this paper demonstrate that multiple specific DNA–protein interactions in the promoter region adjacent to TS2 of the Ad12 E1A gene mediate E1A-independent gene expression. Among these, the sequence which to some extent shows similarity to an E2F-binding site and which is referred to as E2FII was responsible for Ad12 E1A autoactivation, whereas two other binding elements, known to bind ESF-1 (Shibata-Sakurai et al., 1991, 1993) or TBP (Kawamura et al., 1994) were not. Moreover, we present evidence for a synergism in Ad12 E1A autoactivation by the TS1 and the TS2 promoter mediated by E2F-binding sites in both promoter regions.

When fused to a heterologous reporter gene, high TS2 promoter activity in the absence of E1A was obtained only using the construct containing the upstream E2FII motif as well as the palindromic E-Box sequence and its 3′-flanking TATA element. On the one hand, addition of the E2FII motif to the -E2FII/TS2-CAT construct enhanced functional activity by 50%, and conversely gene expression by WT-TS2-CAT was significantly reduced by a half if the E-Box motif was mutated. The mutation affects the E-Box core sequence (Ou et al., 1994) and was previously shown to prevent ESF-1 binding (Shibata-Sakurai et al., 1993; protected sequence 5′ TGTCA; Shibata-Sakurai et al., 1991). From these results we conclude that both promoter elements together with the TATA box contribute to E1A-independent transcription.

By co-transfecting the wild-type TS2 promoter region of the Ad12 E1A gene with an Ad12 E1A 13S expression vector in HeLa, A549 and Raji cells, we found that the region upstream of TS2 could be significantly autoactivated by its own gene product. Considerably enhanced promoter activity was also demonstrated when the E-Box was mutated, whereas in all three cell lines (as well as in transformed HEK cells) neither the E-Box nor the TATA box alone caused a detectable 13S-induced stimulation. These results indicate that the E2F-like motif is responsible for 13S-mediated autoregulation and could confer most of the WT-TS2 transcriptional activity in E1A-expressing cells. Moreover, the moderate increase in transcription observed for the -E2FII/TS2-CAT construct in co-transfected cells (comparable to transformed cells) might be due to an E1A-mediated (Dynlacht et al., 1991) interaction between an E-Box-binding transcription factor, such as ESF-1, and TBP or its associating factor(s), as has been speculated from the loss of in vitro activity when a 7 bp insertion was introduced between both elements (Shibata-Sakurai et al., 1993).

Interestingly, in E1A-non-expressing and 13S-expressing cell lines, the E-Box motif 5′-flanking the TATA element of the TS2 promoter region, which is known to be able to bind the basic helix-loop-helix (bHLH) class of proteins in other promoters (Muré et al., 1989), was found to possess transcriptional activity even if the TATA box was mutated.
(-E2FII/TATAmut/TS2-CAT), suggesting that the E-Box plays a role in specifying the start site of transcription. The TS2 E-Box sequence (5’ CAGCTG) corresponds to the 3’ E-Box flanking the human immunodeficiency virus type 1 TATA box, previously shown to compete with the latter in binding of the TFII D complex (Ou et al., 1994). These results suggest that the E-Box motif may help to regulate the binding affinity of the TFII D complex. Thus, the increase in gene expression mediated by the E-Box (-E2FII/TATAmut/TS2-CAT) in the Ad12 TS2 promoter region is possibly based either on the binding of specific bHLH proteins, which are likely associated with the complex of TBP and TAFs and promote the assembly of the TFII D complex, or TBP which directly binds the E-Box. This is supported by data obtained from gel-shifts, where the E-Box as well as the M2 oligonucleotide with the mutated ESF-1 site are able to compete TATA box complexes (data not shown). Experiments are under way to specify the cellular factor(s) binding to the E-Box element in the E1A gene.

Surprisingly, complexes containing the E2FII sequence in gel-shifts are competed by a specific E2F competitor from the Ad5 E2 gene and blocked by antibodies directed against E2F-5 but not against E2Fs 1, 2 and 4. These data indicate that the E2FII sequence, which shows some similarity to the binding site for E2F when read 3’ to 5’, seems to bind E2F-5 or an E2F-5-like protein which is associated with other cellular factors, like the pocket protein p130. Recent studies indicated that p130 acts as a negative regulator of E2F during G0 and G1 (Shin et al., 1995; Wolf et al., 1995) and that the E2F-5–p130 complex is the most prominent E2F DNA binding species in quiescent cells (Hijmans et al., 1995). Consistently, our results presented here were obtained using nuclear extract prepared from cells grown to 70–80% confluence. Our experiments revealed that E2F-5 is also associated with p107 but not with p105Rb. Since cross-reactivity between these antibodies can be excluded (shown by Santa Cruz, Biotechnology), the effect may probably be due to the closer relationship of p107 to p130 than to p105Rb (Buck et al., 1995; Cobrinik et al., 1993) and may therefore not entirely reflect physiological interactions.

Consistent with our results, previous studies have identified the cellular protein p130 in a complex with E2F as a specific target of the adenovirus E1A (Barbeau et al., 1994; Cobrinik et al., 1993) as well as the simian virus 40 large T antigen during cell transformation (Beijersbergen et al., 1994; Wolf et al., 1995). Inactivation of E2F–p130 DNA binding was shown to correlate kinetically with induction of DNA synthesis and expression of the cellular growth control gene cdk2, whose activation kinetics has been shown to be similar to the E2F-regulated DHFR gene (Dalton, 1992; Slansky et al., 1993; the E2F sites of both cellular genes are shown in Fig. 3). E2F-5–p130 complexes were described to be predominantly present in quiescent cells, and to disappear quickly after cells emerge from quiescence (Hijmans et al., 1995). In this study we demonstrate E1A responsiveness on protein binding to the TS2 E2F-5-like motif. Therefore, we speculate that the TS2 promoter region of the E1A gene is preferentially involved in early responses of resting host cells to the 13S protein, increasing immediately after infection and possibly leading to cell transformation.

However, in contrast to previous reports (Shibata-Sakurai et al., 1991) and with regard to our data described in Kirch et al.
promoter region. Both E2F-1 in the distal (Kirch et al., 1993) and E2F-5 (like) in the proximal region are important for 13S autoregulation. The data suggest an additional synergistic effect for transcription, when the E1A promoter region including TS1 plus TS2 was examined in the presence of viral 13S protein. This effect could be related to the presence of E2F factor binding sites in both promoters and can be explained by an interaction between the 13S-inducible E2F and/or E2F-like proteins. In agreement with previous reports (Saito et al., 1981; Sawada & Fujinaga, 1980) we suppose a 13S-mediated co-operation between both start sites predominantly during early virus infection, while TS2 promoter activation appeared to be of much greater importance in transformed cells. In transformed rat cells, both the 12S and the 13S transcripts from TS2 were found, whereas the longer 12S mRNA, derived from TS1 could not be detected (Sawada & Fujinaga, 1980). Studies are in progress to isolate the cDNA encoding the E2FIII-binding protein.

We thank Phil Gallimore for HEK cells, Bayar Thimmapaya for the kind gift of the EIIA-early WT CAT plasmid, Jane Clifford Azizkhan for the pDHF-210 CAT vector, Ludger Klein-Hitpaß for the anti-Myc antibody, Gerry Ryffel for pCS2MT-ėmyc vector, Dieter Brockmann for the gift of pRC-LTR-CAT and Bertram Opalka for helpful suggestions.

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Received 19 September 1996; Accepted 3 December 1996