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cDNA micro array identification of a gene differentially expressed in adenovirus type 5- versus type 12-transformed cells

Alfred C.O. Vertegaal, H. Bea Kuiperij, Theo van Laar, Volkher Scharnhorst, Alex J. van der Eb, Alt Zantema*

Laboratory for Molecular Carcinogenesis, MGC-Department of Molecular Cell Biology, Leiden University Medical Center, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

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Abstract Proteins encoded by non-oncogenic adenovirus type 5 and oncogenic adenovirus type 12 differentially affect expression of a number of cellular genes. We have used cDNA micro array analysis to identify a cellular gene that is expressed in Ad12- but not in Ad5-transformed cells. This cellular gene was found to be the gene encoding follistatin-related protein, a TGF-β inducible gene. Consistently, a constitutive factor binding to Smad binding elements was found in adenovirus type 12-transformed cells.

Key words: Follistatin-related protein; Adenovirus; Transformation; cDNA micro array; Wilms tumor; TGF-β

1. Introduction

Non-immortal rodent cells and a limited number of non-immortal human cell types can be transformed by human adenoviruses (Ad) [1]. The transforming activity of these viruses maps to the early region 1 (E1) of the viral genome, which encodes the E1A and E1B proteins [2]. A large number of different Ad serotypes exist, which are subdivided on the basis of their capacity to induce tumors in hamsters. Group A consists of highly oncogenic viruses (e.g. Ad12), whereas group C consists of non-oncogenic viruses (e.g. Ad5) [3]. All Ad serotypes are able to transform rodent cells, but only cells transformed by the oncogenic Ad serotypes are oncogenic in syngenic animals. Studies on the differences between cells transformed by Ad5 or Ad12 have led to the identification of differentially expressed cellular genes in the two types of transformed cells, which has contributed to our understanding of oncogenic transformation. The downregulation of MHC class I [4] and the transporters associated with antigen presentation (TAPs) [5,6] in Ad12-transformed cells might be particularly important with respect to the oncogenicity. Due to the lack of MHC class I membrane expression, viral proteins can not be presented to the immune system, which causes these cells to escape immune surveillance by cytotoxic T cells [7].

So far, the mechanism by which Ad5E1 and Ad12E1 lead to differences in gene expression remains unclear, indicating that undiscovered differentially expressed genes must exist that contribute to the downregulation of the MHC I and TAP genes. The recent availability of arrayed ESTs on nylon filters opens a new possibility to screen for differences in gene expression between Ad5- and Ad12-transformed cells. This system enables the identification of differentially regulated genes that are hard to be found by hypothesis-driven strategies. Using this system, we found a novel differentially regulated gene, which encodes the follistatin-related protein (FRP). Previously, FRP was found to be a target for TGF-β and in accordance with this finding, a constitutive factor binding to Smad binding elements (SBE) was found in Ad12-transformed cells.

2. Materials and methods

2.1. Materials

EST W52072 was obtained from Incyte Genomics (St. Louis, MO, USA). ESTs AI690397 and AI866922 were obtained from the Resource Center/Primary Database of the German Human Genome Project, Max Planck Institute (Berlin, Germany). TGF-β1 was obtained from R&D systems (Minneapolis, MN, USA).

2.2. Cell lines and culture

Human embryo cells from retina (HER), kidney (HEK) and lung (HEL) cells were cultured in F-15 medium supplemented with 10 or 15% FCS and antibiotics. For EMSA experiments, cells were grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics. Furthermore, 1 mM pyruvate, 15 mM glucose, 4.1 mM L-glutamine and 8.2 μM biotin were added. Tissue culture media and sera were purchased from Gibco BRL (Gaithersburg, MD, USA). All tissue culture-plastics were obtained from Greiner (Solingen, Germany).

2.3. Comparative cDNA micro array analysis

cDNA micro array analysis was performed using the human I version 1.2 Gene Discovery Array from Incyte Genomics (St. Louis, MO, USA) according to the instructions of the manufacturer. This array consists of 18 376 ESTs arrayed on nylon filters. [α-^{33}P]dCTP-labeled complex cDNAs were prepared from mRNA of exponentially growing Ad5-transformed HER clone 1 and Ad12-transformed HER clone 7 cells. These two cDNA pools were used to hybridize two identical arrays. Signals were quantified by phosphorimager and subsequently, these arrays were stripped and hybridized with the other cDNA pool.

2.4. RNA extraction and Northern blotting

Total RNA was isolated from exponentially growing cells as described previously [8]. 10 μg of total RNA from exponentially growing cells was size-fractionated on a 1% agarose/2.2 M formaldehyde gel by electrophoresis and transferred to Hybond filters (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Filters were hy-

*Corresponding author. Fax: (31)-71-527 6284.
E-mail: zantema@lumc.nl

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bridized with the indicated probes. A human multiple-tissue Northern blot (7780-1; Clontech, Palo Alto, CA, USA) was hybridized according to the instructions of the manufacturer. RNA was isolated from Wilms tumors and corresponding normal kidney tissue as described previously [9].

2.5. Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts, binding reactions and electrophoresis were essentially performed as described elsewhere [8]. Where indicated, the cells were treated with 7 ng/ml TGF-β1 for 30 min in DMEM supplemented with 0.3% FCS and antibiotics. Nuclear extracts were incubated with a double-stranded oligonucleotide containing three SBE. The sequence of the double-stranded SBE oligonucleotide was 5′-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAG-3′ and the complementary strand 5′-GTGTCTGGCTCCTTGTCTGGCTC-3′. This double-stranded oligonucleotide was labeled by filling in recessed 3′ ends with [α-^{32}P]-dATP and [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. Binding reactions containing 10 μg of nuclear extracts and 25 fmol of labeled oligonucleotides were performed for 30 min at room temperature in 50 μl of binding buffer. For competition experiments, a 33-fold molar excess of cold competitor probe was added. The sequence of the double-stranded SBE mutant oligonucleotide used in these competition experiments was 5′-TCGAGAGCTACATAAGGAGCTACATAAGGAGCTAC-3′ and the complementary strand 5′-GTATGTAGCTCCTTATGTAGCTCCTTATGTAGCTC-3′, made completely double-stranded by filling in the recessed 3′ ends using the Klenow fragment of DNA polymerase I. After electrophoresis, the 5% polyacrylamide gels were dried and exposed to X-ray films.

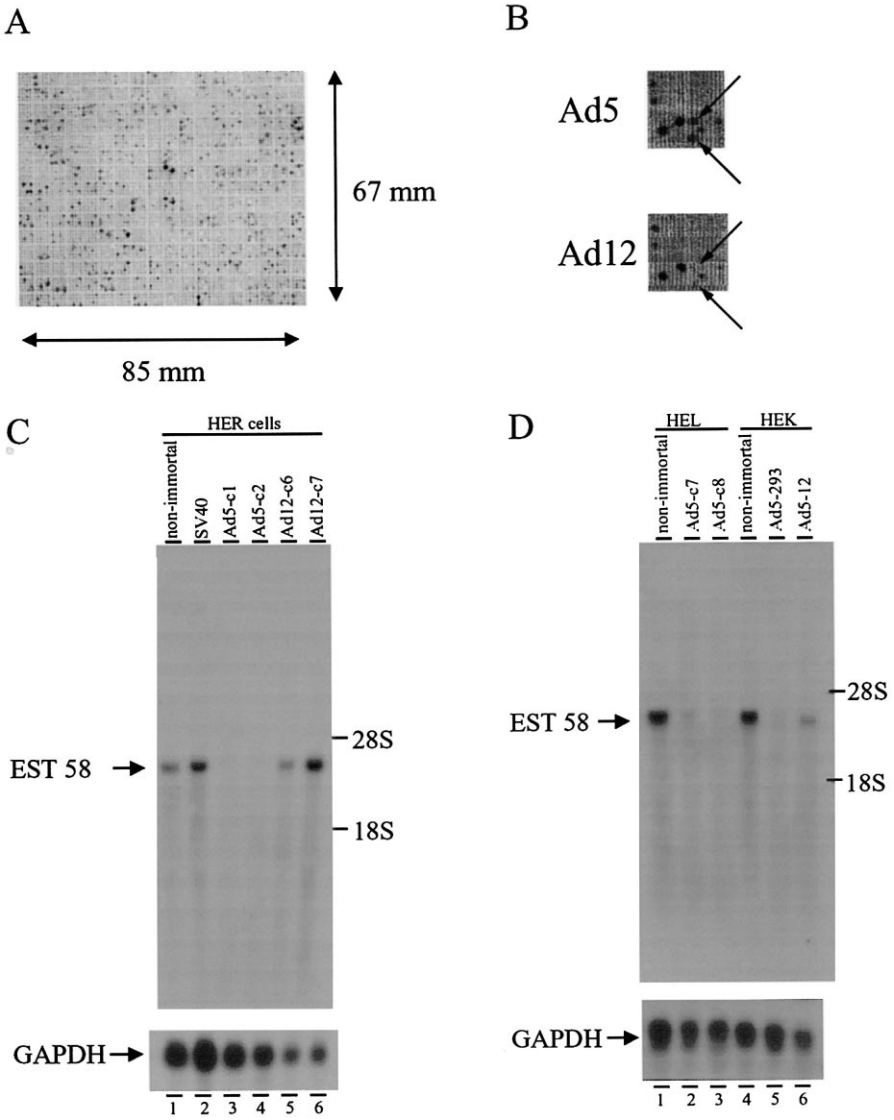

Fig. 1. Differential expression of the gene corresponding to EST58 in Ad5 and Ad12-transformed cells. A: Gene expression profiling of Ad5- and Ad12-transformed cells using cDNA micro arrays. Shown is a part of the gene expression profile of Ad5-transformed cells. B: Detail of cDNA micro arrays. Differentially expressed genes were detected by comparing the intensity of signals between both micro arrays. Shown is a small fragment of the two micro arrays. Arrows indicate a gene (spotted twice) that is more highly expressed in Ad5 cells than in Ad12 cells. C: Differential expression of the gene corresponding to EST58 in Ad5- and Ad12-transformed HER cells. Equal amounts of total RNA from exponentially growing HER cells was size-fractionated by electrophoresis and transferred to Hybond filters. Filters were hybridized with [α-^{32}P]dCTP labeled 0.8 kb *Not*I–*Eco*RI EST58 fragment. Indicated are the 28S and 18S ribosomal RNA bands. Control hybridizations were performed using the *Pst*I fragment of the rat GAPDH cDNA. D: Downregulation of the gene corresponding to EST58 in Ad5-transformed HEL- and HEK cells. Northern blot analysis was performed as described under C.

3. Results and discussion

3.1. Comparative cDNA micro array analysis of Ad5- and Ad12-transformed cells

To study differences in gene expression between Ad5- and Ad12-transformed cells, partial gene expression profiles were obtained using the human I version 1.2 Gene Discovery Array from Incyte Genomics. Fig. 1A shows a part of the gene expression profile of Ad5-transformed HER clone 1 cells. In these hybridizations, 11 genes were consistently found to be differentially expressed in the Ad5-and Ad12-transformed HER cells. One example of a gene that is expressed more highly in Ad5-transformed cells than in Ad12-transformed cells is shown in Fig. 1B. The corresponding ESTs of these 11 genes were obtained and used as probes in Northern blot analysis. Five out of the 11 genes turned out to be indeed differentially expressed in these Ad5- and Ad12-transformed cell lines. Equal expression was found for two genes, no signal could be detected for two other genes and hybridization with two probes resulted in a smear, probably due to the presence of repeat sequences (data not shown). Using a set of Ad5- and Ad12-transformed clonal cell lines, consistent differential expression of one of the five genes was found (Fig. 1C).

The detection of only one consistently differentially expressed gene is surprising as several genes have been reported to be differentially expressed in Ad5- versus Ad12-transformed cells. Of the differentially expressed genes, MHC I [4], $p90^{rsk1}$ [10] and JunB [11] ESTs were present on the filter. However, not all ESTs turned out to have the correct sequence. In the course of various array projects in our laboratory, we resequenced 47 ESTs used for the human I version 1.2 Gene Discovery Array and found that the sequence of 15 ESTs was completely different from the indicated sequences, showing the low reliability of the given EST sequences. For two additional ESTs of the set of 47 ESTs, clones were obtained that consisted of mixtures of two different plasmids. The sequences that were determined for the insert of these plasmids also differed from the sequences given by Incyte Genomics. Thus the overall reliability of this set of ESTs was 64%. Nevertheless, we paid special attention to the ESTs that could be expected to show a difference in Ad5- and Ad12-transformed cells. One EST corresponding to MHC class I was found to be indeed differentially regulated in the micro array analysis (data not shown), but the difference in expression was rather weak, which explains why we had ignored this EST. A weak $p90^{rsk1}$ signal was detected for both cell lines of comparable intensity (data not shown). At the position of the JunB-corresponding EST, as indicated by Incyte Genomics, no signal was detected. Many of the regulatory proteins like kinases and transcription factors, are known to be expressed at very low levels [12]. The fact that for the mRNA of the transcription factor JunB no signal could be detected and for the kinase $p90^{rsk1}$ only a weak signal was found, combined with the finding that on average a signal could be detected for only 10% of the ESTs (Fig. 1A), indicates a rather low overall sensitivity of the assay. This is a general problem for micro arrays, also when glass slides are used instead of nylon filters [13].

The differentially expressed gene that we found corresponds to EST58 (our nomenclature). A probe derived from this EST detected significantly lower mRNA levels in Ad5-transformed HER cell lines than in Ad12-transformed HER cell lines (Fig. 1C). Compared to non-immortal HER cells and an SV40-transformed HER cell line, expression of the gene corresponding to EST58 was decreased in Ad5-transformed HER cell lines and, assuming constant GAPDH levels, increased in Ad12-transformed HER cell lines. Decreased expression was also found in Ad5-transformed HEL and HEK cell lines, compared to non-immortal cells (Fig. 1D). We have been unable to transform these cell types with the E1 region of Ad12. However, non-immortal HEK cells have been transformed with a hybrid construct containing Ad5E1A and Ad12E1B. Expression of EST58-corresponding mRNA is still significantly lower in this cell line compared to non-immortal cells, although not as low as in the Ad5-transformed cell line. This suggests a role for Ad5E1A in the process of downregulation of the EST58 gene (Fig. 1D).

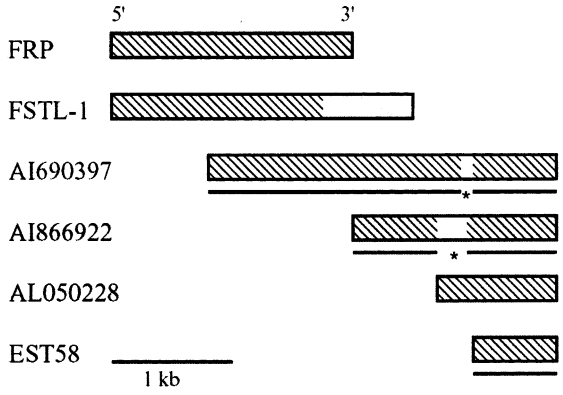

Fig. 2. Sequence analysis of EST58 and overlapping ESTs. EST58 and the underlined parts of ESTs AI690397 and AI866922 were sequenced. These sequences were completely identical to the overlapping sequence of the gene encoding the FRP and partially identical to the overlapping sequence of the gene encoding FSTL-1. Hatching indicates identical overlapping sequences. The gray box indicates the part of FSTL-1 that differed from our overlapping sequence and the overlapping sequence of FRP. Asterisks indicate parts of ESTs that have not been sequenced.

3.2. EST58 corresponds to the gene encoding FRP

According to the sequence information of Incyte Genomics, EST58 is W52072. We resequenced this EST and found that its sequence was completely different. A BLAST search did not reveal any homology to other sequences deposited in GenBank [14]. Later on during this project, several other ESTs with overlapping sequences were identified in GenBank and subsequently obtained. The 5′ and 3′ parts of these ESTs were sequenced (Fig. 2) and the sequence of the 5′ 2180 bp of AI690397 is deposited in GenBank (accession number AF288537). An open reading frame was found in the most 5′ part of AI690397 which was identical to the overlapping sequence of the gene encoding the FRP [15]. Tanaka et al. have published an independently isolated clone, which was named follistatin like-1 (FSTL-1) [16]. FSTL-1 differs from our sequence and from the published FRP sequence in the 3′ part of the untranslated region. Searching the human genome database revealed that only this part of the FSTL-1 sequence maps to chromosome 7, whereas sequence-tagged sites of FRP and EST58 map to chromosome 3. This indicates that the published FSTL-1 sequence might be derived from a hybrid cDNA originating from FRP and a second transcript. The conclusion is that the gene corresponding to EST58 is *frp*.

Consistently, rehybridization of the Northern blots of Fig. 1 with a 5′ probe from clone AI690392 gave identical results (data not shown).

3.3. Expression of FRP in normal and transformed tissues

To study the expression levels of FRP in normal human tissues, a multiple-tissue Northern blot was hybridized with a probe corresponding to the 3′ end of FRP (Fig. 3A). For most tissues, a single transcript was detected of approximately

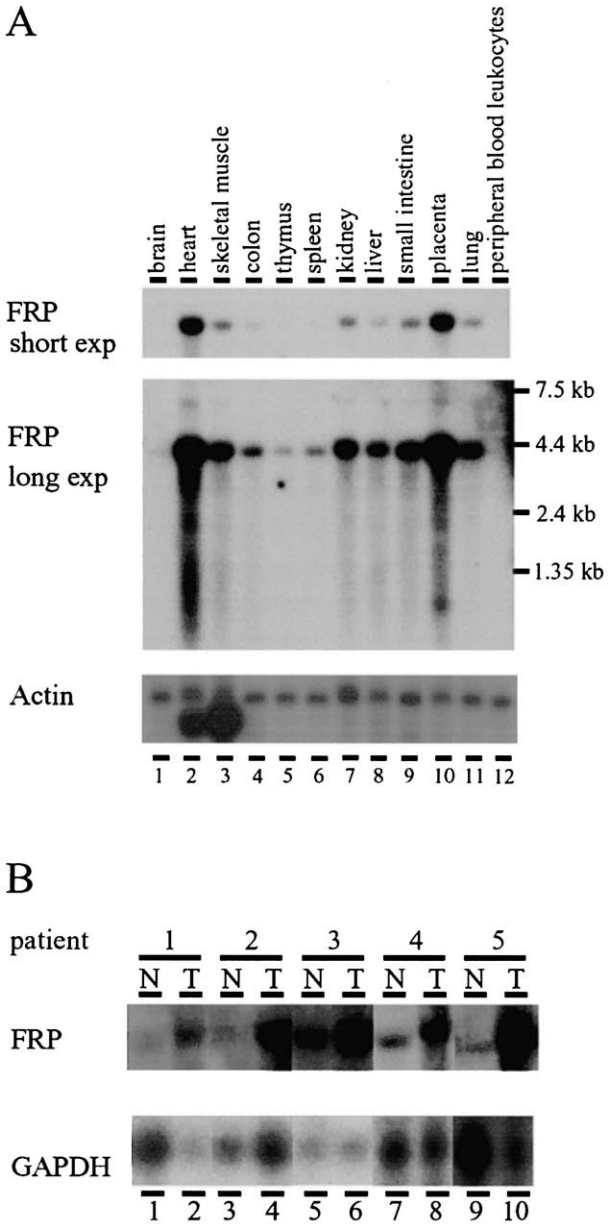

Fig. 3. mRNA levels of FRP in normal and transformed tissues. A: A multiple-tissue Northern was hybridized with a [α-^{32}P]dCTP labeled 0.8 kb *Not*I–*Eco*RI EST58 fragment. As a control for equal loading, the expression of β-actin was determined. B: FRP mRNA is upregulated in Wilms tumors. Total RNA was isolated from Wilms tumors (T) and corresponding normal kidney tissue (N). Equal amounts of total RNA were size-fractionated and transferred to Hybond filters. These filters were hybridized with a [α-^{32}P]dCTP labeled 0.8 kb *Not*I–*Eco*RI EST58 fragment. Control hybridizations were performed using the *Pst*I fragment of the rat GAPDH cDNA.

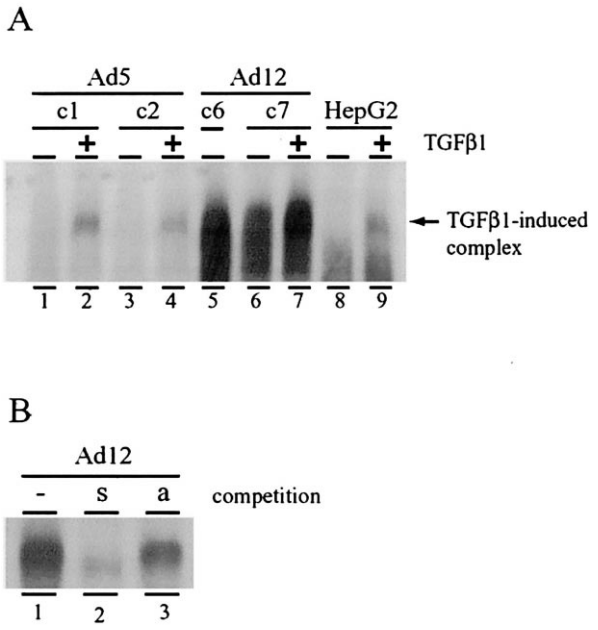

Fig. 4. EMSA for binding of Ad5- and Ad12 nuclear proteins to SBE. A: Nuclear extracts of TGF-β1-treated (lanes 2, 4, 7 and 9) or untreated (lanes 1, 3, 5, 6 and 8) exponentially growing Ad5-HER clone 1 (lanes 1 and 2), – clone 2 (lanes 3 and 4), Ad12-HER clone 6 (lane 5) – clone 7 (lanes 6 and 7) and HEPG2 cells (lanes 8 and 9) were incubated with a double-stranded oligo containing three SBE. TGF-β1-induced complexes are indicated. The specificity of this band is shown in (B), where nuclear extracts of untreated exponentially growing Ad12-HER clone 7 were incubated with the labeled probe as described in (A, lane 1) in the presence of cold specific (s) competitor (lane 2) or a-specific (a) competitor (lane 3).

4 kb and upon prolonged exposure, an additional longer transcript was detected. FRP was highly expressed in heart (lane 2) and placenta (lane 10), low expression was found in colon (lane 4), thymus (lane 5) and spleen (lane 6) and no expression could be detected in brain (lane 1) and peripheral blood leukocytes (lane 12). These results are largely similar to those of Tanaka et al., except that they do detect expression of FRP in the human brain [16].

Since Ad12 transformation has been shown to be a suitable model for oncogenic transformation, we studied whether FRP expression is also deregulated in human tumors. Upon hybridization of Northern blots with the FRP cDNA probe described above, a significant increase in FRP expression in Wilms tumors compared to primary kidney tissue was observed in five out of five patients (Fig. 3B).

It has been suggested that FRP functions as a TGF-β antagonist [17]. This hypothesis is mainly based on the presence of a domain that was previously found in follistatin, an inhibitor of the TGF-β family members activin and BMPs [18]. Contrary to this hypothesis, FRP has been found to have no effect on the growth-inhibitory action of TGF-β1 on CCl-64 cells [15]. Furthermore, it does not bind to TGF-β1, BMP4 or BMP7 [19]. Clearly, a better understanding of the biological function of FRP will be necessary to determine which role FRP may have in Ad transformation.

3.4. FRP expression correlates with factor binding to SBE

FRP was originally identified as a TGF-β-regulated gene [17]. Therefore, the difference in FRP expression between Ad5- and Ad12-transformed HER cell lines might be ex-

plained by a difference in TGF-β signaling between these cell lines. TGF-β binds to its receptor, which leads to phosphorylation and thereby activation of Smad, which subsequently translocate to the nucleus and activate transcription of target genes by binding to SBE in the promoters of these genes [18]. To test whether Ad5- and Ad12-transformed HER cell lines differ in their factor binding activity to SBE, an EMSA was carried out (Fig. 4A). In untreated Ad5-transformed HER cells and in control HepG2 cells [20], a signal is hardly visible at the position of the retarded band induced by TGF-β (Fig. 4, lanes 1–4, 8 and 9). In contrast, in Ad12-transformed HER cell lines (lanes 5 and 6) a strong retarded band is already present in the absence of TGF-β stimulation (lanes 5 and 6), which is further induced by TGF-β treatment (lane 7 versus 6). Therefore, in Ad12-transformed HER cell lines a constitutive binding activity to SBE is present. This binding activity is specific for the SBE elements, since it can be competed with a 33-fold molar excess of unlabeled wild-type SBE probe, but not with unlabeled mutant SBE probe (Fig. 4B). The mechanism leading to enhanced factor binding is presently not known. An obvious explanation of constitutive expression of active TGF-β forms by Ad12-transformed cells turned out to be unlikely as both Ad5- and Ad12-transformed HER cells secrete inactive TGF-β that upon activation by heat treatment has a comparable activity (data not shown). Also the significance of binding activity to SBE in Ad12-transformed cells is not known, but it is reasonable to assume that it will lead to a differential expression of genes normally regulated by the TGF-β family. This differential gene expression might well contribute to the differences in oncogenic behavior of Ad5- and Ad12-transformed cell lines, as the TGF-β factors are known to play essential roles in growth and development [18].

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