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PolyADP-ribose polymerase is a coactivator for AP-2-mediated transcriptional activation

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ABSTRACT

Overexpression of transcription factor AP-2 has been implicated in the tumorigenicity of the human teratocarcinoma cell lines PA-1 that contain an activated *ras* oncogene. Here we show evidence that overexpression of AP-2 sequesters transcriptional coactivators which results in self-inhibition. We identified AP-2-interacting proteins and determined whether these proteins were coactivators for AP-2-mediated transcription. One such interacting protein is polyADP-ribose polymerase (PARP). PARP suppresses AP-2 self-inhibition and enhances AP-2 activity in PA-1 cells indicating that it is a coactivator for AP-2-transcription. PARP significantly restores AP-2 transcriptional activity in *ras* oncogene-transformed cells suggesting that it might suppress transformation in these cells. Another AP-2-interacting protein, RAP74, a subunit of transcription factor TFIIF, does not affect AP-2-mediated transcriptional activation alone or in the presence of RAP30, the other subunit of TFIIF. RAP74 also fails to relieve AP-2-mediated transcriptional self-interference and cross-interference. These studies suggest that the interaction between AP-2 and RAP74 may have functions other than activation of AP-2-mediated transcription.

INTRODUCTION

Many transcription factors are DNA-binding proteins that recognize *cis*-regulatory elements of target genes and are the most direct regulators of gene transcription (1). Activation of signal transduction pathways results in changes in transcription factors to alter gene expression (2). The regulation of cell growth and differentiation is controlled by transcription factors and mechanisms that modulate their activity at various levels, including transcription, post-transcription, translation, post-translation, ligand binding and interaction with other proteins (1). The aberrant regulation of transcription factors is often associated with pathological conditions of a cell. We find that activation of *ras* oncogene results in deregulation of the transcription factor AP-2 which is the critical mechanism of oncogenic transformation of PA-1 cells (3). We have also demonstrated that overexpression of AP-2 in non-*ras* PA-1 cells results in transformation similar to *ras* PA-1 cells. Detailed analysis of AP-2-mediated transformation revealed that overexpression of AP-2 resulted in transcriptional self-interference and inhibition of its activity (3). Preliminary evidence suggested that sequestration of intermediary factors might be the cause of their self-interference. In this report we find that when AP-2 expression is high it interferes with the transcriptional activities of different activators VP16 and SRF via a mechanism of sequestration of common coactivators. The mechanism of sequestration of coactivators leads to tumorigenicity and it is probable the coactivators that relieve AP-2 transcriptional self-interference can suppress cellular transformation induced by AP-2 and *ras* oncogene.

We sought to identify AP-2 interacting proteins and determine whether those proteins were coactivators for AP-2-mediated transcription. We identified three proteins that physically interacted with AP-2. A 19 kDa protein was identified as the positive coactivator PC4 (4). Further experiments indicated that PC4 is a coactivator for AP-2-mediated transcription. PC4 was capable of relieving AP-2 transcriptional self-interference, but not completely. Preliminary experiments suggested that more than one coactivator is affected by AP-2 overexpression. We report here the identification of two other proteins that interact with AP-2; polyADP-ribose polymerase (PARP) and the RAP74 subunit of transcription factor TFIIF. PARP is a nuclear enzyme that catalyzes the transfer of ADP-ribose units from NAD^+ to nuclear protein acceptors (5,6). PARP associates with other macromolecules and participates in several cellular catalytic activities (7), including DNA replication (8), HIV replication (9), DNA repair (5) and carcinogenesis (7,10). PARP associates with transcription factor p53 (11) and is hypothesized as a participant of the p53-mediated G_1 arrest signal transduction pathway through the modulation of WAF-1/CIP1/p21 and MDM-2 mRNA expression (12). Cleavage of PARP into DNA-binding and catalytic fragments serves as a sensitive parameter for identification of different types of cell death and as a marker for activation of a cell death protease (13). Slattery *et al.* (14) identified PARP as the basal transcription factor TFIIC in their earlier studies. PARP co-purified with a number of transcription factors and was thought to participate in basal transcription. However, their experiments indicated that PARP was not required in basal transcription in systems reconstituted with purified factors. A recent study by the same group shows that PARP is a

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transcriptional coactivator that enhances GAL4-AH and NFκB TA1 activator-dependent transcription *in vitro* (15). The RAP74 subunit of transcription factor TFIIF is a 74 kDa protein, a component of the general cellular transcriptional machinery that consists of RNA polymerase II, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (16). RAP74 has been shown to be a coactivator for SRF- and VP16-mediated transcription (17,18). RAP74 relieves SRF and VP16 self- and cross-interference *in vitro*. Here, we analyzed the activity of PARP and RAP74 as transcriptional coactivators that enhance AP-2-mediated transcription and investigated whether they could relieve AP-2 transcriptional self-interference in *ras* oncogene-transformed cell lines.

MATERIALS AND METHODS

Cell culture

PA-1 human teratocarcinoma cells were derived from a female ovarian germ cell tumor (19); the origin and properties of non-*ras* and *ras* PA-1 sublines were described previously (20). The cells were grown in modified Eagle's medium with Earl's salts (Gibco Laboratories, Gaithersburg, MD) supplemented with 5% fetal bovine serum (Hazelton Biologics, Lenexa, KS) and antibiotics at 37°C in 5% CO_2, 95% air. The MDA-MB453 mammary carcinoma cell line was grown in similar conditions with Iscove's modified Dulbecco's medium with 10% fetal bovine serum.

Analysis of GST–AP-2-associated proteins

The GST–AP-2 binding assays were performed to identify the proteins that specifically associated with AP-2. Nuclear extracts were prepared essentially as previously described (21) from metabolically ^{35}S-labeled PA-1 cells. An aliquot of 8×10^6 TCA-precipitable counts of nuclear extracts was mixed with 20 µg of bacterial GST–AP-2 protein that was purified as described (22) and bound to glutathione–Sepharose beads. After rocking for 2 h at 4°C the mixture was washed four times in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST). AP-2 and its associated proteins were released by using 0.02 U of blood coagulation factor Xa (Boehringer Mannheim Corp., Indianapolis, IN) in 1 mM $CaCl_2$ and incubating at 25°C for 1 h. AP-2 and its associated proteins were boiled in SDS loading buffer and resolved on a 10% polyacrylamide gel (23). The gel was dried and exposed to Kodak X-OMAT X-ray film. When cold PA-1 nuclear extracts were used in the studies, the proteins were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Corp., Arlington Heights, IL) and probed with a mouse monoclonal antibody against PARP (a gift of Dr G.Poirier) or rabbit polyclonal antiserum raised against RAP74 (a gift of Dr R.Prywes). The signals were detected by using anti-mouse HRP or anti-rabbit HRP antibody and electrochemiluminescence (Amersham Corp.) according to the manufacturer's instructions.

Expression plasmids

Expression plasmid pSAP2 containing AP-2 (AP-2α) cDNA in the plasmid pSG5 (Stratagene, La Jolla, CA) and the GAL4 DNA-binding domain and AP-2 activation domain fusion construct pGAL4-AP-2/11–226 were described previously (3). Human PARP cDNA (a gift of Dr Bürkle) and human AP-2γ (a gift of Dr Hurst) were subcloned into pSG5 that was cut with *Eco*RI and blunted by filling in with Klenow polymerase to generate pSPARP and pSAP-2γ. PSG5 contains an SV40 early promoter and β-globin intron sequences that enable efficient expression of cloned genes. pCMXAP-2β that contains murine AP-2β cDNA was a gift of Dr Buettner. GST–AP-2/1–165, GST–AP-2/1–277, GST–AP-2/ΔN 165 and GST–AP-2/ΔI 166–278 fusion proteins were made from the previously described GST–AP-2 fusion protein (24) by deleting sequences from the *Sma*I site at amino acid 165 and/or the *Pst*I site at amino acid 278. All these constructs were verified for their nucleotide sequence and reading frame by double-stranded DNA sequence analysis.

Immunoprecipitation

Immunoprecipitation studies were carried out to analyze the interaction of PARP and RAP74 with AP-2. The presence of T7 promoter in pSG5 and pCMX vectors enables *in vitro* transcription and translation of cloned PARP and various forms of AP-2 genes. *In vitro* synthesis of proteins was performed using the TNT *in vitro* transcription and translation system (Promega Corp., Madison, WI) according to the manufacturer's instructions with 2 µg of plasmid DNA and 40 µCi of [^{35}S]L-methionine in a 50 µl reaction. Immunoprecipitation was carried out in 1 ml of TBST using 2 µl of antibody and 20 µl of protein A adsorbed to agarose beads for 4 h at 4°C. The immunoprecipitated complex, after washing four times in TBST, was boiled in SDS sample loading buffer and resolved on a 10 or 14% SDS–polyacrylamide gel. The ^{35}S-labeled protein signal was amplified using Amplify (Amersham Corp.) and dried under vacuum and exposed to Kodak BIOMAX MR X-ray films at –80°C.

Interaction studies of cellular PARP and AP-2 proteins were carried out with nuclear extract of the MDA-MB453 mammary carcinoma cell line that overexpresses AP-2α. An aliquot of 1 mg of nuclear extract in 1 ml of TBST and 2 µl of AP-2 polyclonal antibody specific for AP-2α (Serotec, Raleigh, NC) was used for immunoprecipitation as above. The proteins were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Corp.) and probed with a PARP antibody (Serotec). The signals were detected by using anti-mouse HRP antibody as above.

Transient transfections of PA-1 cells and CAT assays

Transient transfections using calcium phosphate precipitation as described previously (25) were performed to introduce DNA into PA-1 cells. The amount of DNA used in all transfections was equalized by the addition of pBluescript DNA. The *trans*-activation activity of various activators was determined by measuring the CAT activity using respective expression plasmids and reporter constructs as follows. AP-2 response element sequences from the distal basal level element of the human metallothionein IIa gene corresponding to nucleotides –188 to –161 were oligomerized and a reporter construct 3× AP-2–CAT was made by cloning three response elements adjacent to the HSV *tk* promoter in the vector pBLCAT2 (26). GAL4–VP16 expression plasmid pSGVP and GAL4 reporter plasmid G5E1bCAT were generous gifts of Dr Ptashne. Adenovirus major late promoter (AMLP) linked to a CAT gene was a gift of Dr Sawadago (M. D. Anderson Cancer Center, Houston, TX). The serum response factor (SRF) expression plasmid pCGNSRF and its reporter construct pFC700 were gifts of Dr Prywes (Columbia University, New York, NY). CAT activity normalized in 10–20 µg proteins was measured by the conversion of [^{14}C]chloramphenicol to monoacetyl- and diacetyl-chloramphenicol, essentially as described earlier (27). After

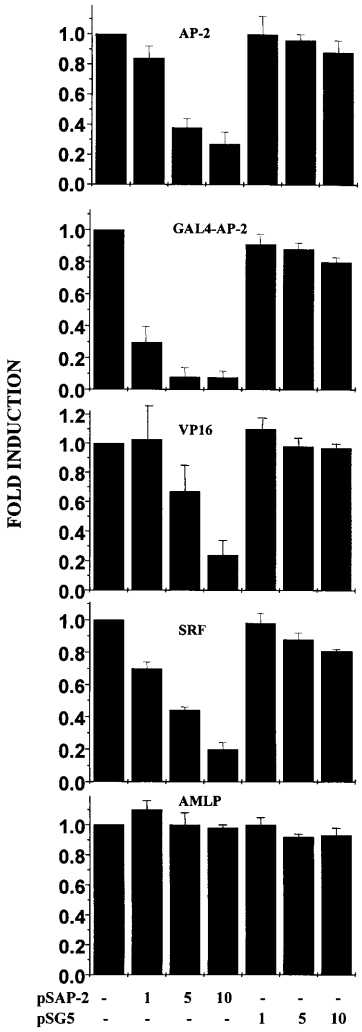

Figure 1. AP-2 inhibits the activities of GAL4–AP-2, GAL4–VP16 and SRF. Experiments were carried out as described in Materials and Methods. The indicated amounts of pSAP2 (or the control plasmid pSG5) were co-transfected with 4 µg of 3× AP-2–CAT reporter to measure the activity of AP-2 or 1 µg of pGAL-P-2/11–226 and 4 µg G5E1bCAT reporter plasmid to measure the activity of GAL4–AP-2 or 0.5 µg of pSGVP and 2 µg of G5E1bCAT GAL4 to measure the activity of VP16 or 4 µg of fosCAT to measure the activity of SRF or 4 µg of pAMLPCAT to measure the activity of AMLP. The activities of AP-2, GAL4–AP-2, VP16, SRF and AMLP in the absence of co-transfection of AP-2 expression plasmid pSAP-2 is taken as 1 and compared with other transfections.

partitioning the acetylated forms of chloramphenicol on thin-layer chromatography the percentage conversion was calculated by measuring radioactivity on a Storm analyzer (Molecular Dynamics Inc., Sunnyvale, CA). The experiments were repeated two to four times for each assay and the variation in the assays which did not exceed >35% are shown with error bars in Figures 1, 3 and 5. Plasmid pCH110 (Pharmacia Biotech., Piscataway, NJ) that contains the *lacZ* gene under the control of an SV40 promoter was used to test the effect of PARP on SV40 promoter. β-Galactosidase enzyme activity was determined as described (28).

In vitro transcription

Plasmid pcmyc-PC is a derivative of pC2AT (29), which contains the human c-*myc* P2 promoter from nt –44 to +4 and a 398 bp G-free transcription cassette. Three AP-2 sites found in the human metallothionein IIa gene basal level promoter from nt –188 to –159 were cloned upstream of c-*myc* P2 promoter to create pcmyc-AP-2. *In vitro* transcription reactions were performed using 50 ng of plasmid DNA, 10 µCi [α-^{32}P]UTP and the HeLa Cell Extract Transcription System (Promega Corp., Madison, WI) essentially as described by the manufacturer. Bacterial AP-2 protein was purified from His-tagged AP-2 fusion protein using Ni^{2+} columns according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). PARP protein was a gift of Dr Poirier (30). The transcription products were separated on a 5% polyacrylamide gel containing 7 M urea, dried and exposed to Kodak BIOMAX MR film at –70°C. Plasmid p052 was used as a control plasmid in the *in vitro* transcription reactions. This control plasmid contains an unrelated *hsp70* promoter, a 298 bp G-free transcription cassette and a weaker, mutated form of adenovirus major late initiator (31).

RESULTS

Overexpression of AP-2 interferes with the activities of GAL4–AP-2 and GAL4–VP16 fusion proteins and SRF

Overexpression of AP-2 self-interferes with its activity (3). Transformation of PA-1 cells by an activated *ras* oncogene also induces AP-2 self-inhibition. AP-2 self-interference occurs independent of AP-2-specific DNA binding as AP-2 can interfere with GAL4–AP-2 fusion protein that contains the activation domain of AP-2 fused to a heterologous DNA-binding domain of GAL4. GAL4 is a yeast transcription factor involved in galactose metabolism with no natural target DNA-binding sites in normal human cellular DNA. As the activation domain was sufficient for self-interference it appeared that AP-2 may be sequestering coactivators needed for its activity. To confirm whether sequestration of coactivators is the cause for AP-2 transcription interference we tested the effect of AP-2 overexpression on other activators with different target DNA specificity. The GAL4–VP16 fusion protein contains a Herpes simplex viral transcriptional activator fused to the DNA-binding domain of GAL4. SRF binds to serum response elements (SREs) found in the regulatory region of the c-*fos* proto-oncogene, a number of growth factor-inducible genes and muscle-specific genes (32). Transient transfection experiments were performed to measure the *trans*-activation properties of these activators. The activity of each factor alone was used to normalize the respective fold induction. Overexpression of AP-2 inhibits the activity of GAL4–VP16 and SRF in a dose-dependent manner (Fig. 1). In co-transfection experiments, 10 µg of AP-2 expression plasmid was able to inhibit the activity generated by 1 µg of GAL4–VP16 expression plasmid by ~4-fold. At the same concentration, the vector control inhibited of the GAL4–VP16 activity by only 3%. Similarly, the endogenous SRF activity of PA-1 cells was inhibited by ~5-fold when 10 µg of AP-2 expression plasmid was transfected into these cells. An aliquot of 10 µg vector control inhibited SRF activity by <20%. These experiments suggest that AP-2, VP16 and SRF share common coactivators and overexpression of AP-2 sequesters these factors. As the activity of AMLP was not significantly affected by the overexpression of AP-2, the inhibition of the activities of

GAL4–VP16 and SRF by AP-2 was not due to a general inhibitory effect on cellular transcription.

Identification of proteins interacting with AP-2

The above experiments indicated that AP-2 protein binds and sequesters coactivators. In order to identify such coactivators we sought to find AP-2-interacting proteins in nuclear extracts of PA-1 cells and then analyze their function. A GST–AP-2 fusion protein that was adsorbed to glutathione–Sepharose beads was allowed to interact with ^{35}S metabolically labeled nuclear extracts of PA-1 cells and AP-2-associated proteins were visualized (Fig. 2A). A number of polypeptides were seen specifically associated with AP-2. We focused on one prominent polypeptide of ~110 kDa and two other polypeptides of ~74 and 19 kDa. The association of these three polypeptides was not seen when GST alone was used in the assays. Two polypeptides, both ~60 kDa, also interacted with AP-2; however, the reproducibility of their association was inconsistent under these experimental conditions.

We have shown earlier that the 19 kDa protein is indeed the previously known positive coactivator PC4 (4). Due to the complication of background polypeptides (that also interacted with bacterial GST protein) the natures of the 74 and 110 kDa polypeptides were identified by a combination of approaches. We searched for previously known coactivators with similar molecular weights. The RAP74 subunit of TFIIF relieves the SRF- and VP16-mediated transcriptional self-interference (17,18). As shown above, AP-2 interferes with the activity of SRF and VP16. The molecular weight of RAP74, 74 kDa, matched one of the polypeptides identified in the nuclear extracts of PA-1 cells that specifically bound GST–AP-2 (Fig. 2A). To demonstrate a RAP74 interaction with AP-2, unlabeled nuclear extracts of PA-1 cells were prepared and GST–AP-2 binding assays were performed as described above. The AP-2-bound proteins were resolved on a SDS–polyacrylamide gel and western blot analysis was performed by probing with a RAP74-specific antibody. As shown in Figure 2B, the RAP74 antibody recognized a 74 kDa polypeptide that showed specific binding to GST–AP-2 but not to GST.

Overexpression of c-*erbB2*/HER2 is regulated by transcription factor OB2-1 in mammary carcinoma (33). The transcription factor OB2-1 was later found to be identical to transcription factor AP-2. Antibodies raised against a non-homogeneous purification of OB2-1 protein also contained antibodies to a 110 kDa protein, indicating that these two proteins, of 52 (OB2-1) and 110 kDa, co-purified. Co-purification suggested that these two proteins were strongly associated with each other. Partial sequence analysis identified the 110 kDa polypeptide as PARP (34). PARP is an extensively studied enzyme that polyADP-ribosylates chromatin proteins (6,7,35). PARP associates with a number of proteins and participates in many biological functions (7). As OB2-1 (AP-2) interacted with a 110 kDa polypeptide, we analyzed whether PARP could interact with AP-2. A western blot as above containing the GST–AP-2-interacting nuclear proteins was probed with a monoclonal antibody raised against PARP. As shown in Figure 2C, the PARP monoclonal antibody recognized a 110 kDa protein among the proteins that interacted with GST–AP-2. These observations indicated that RAP74 and PARP physically interact with AP-2.

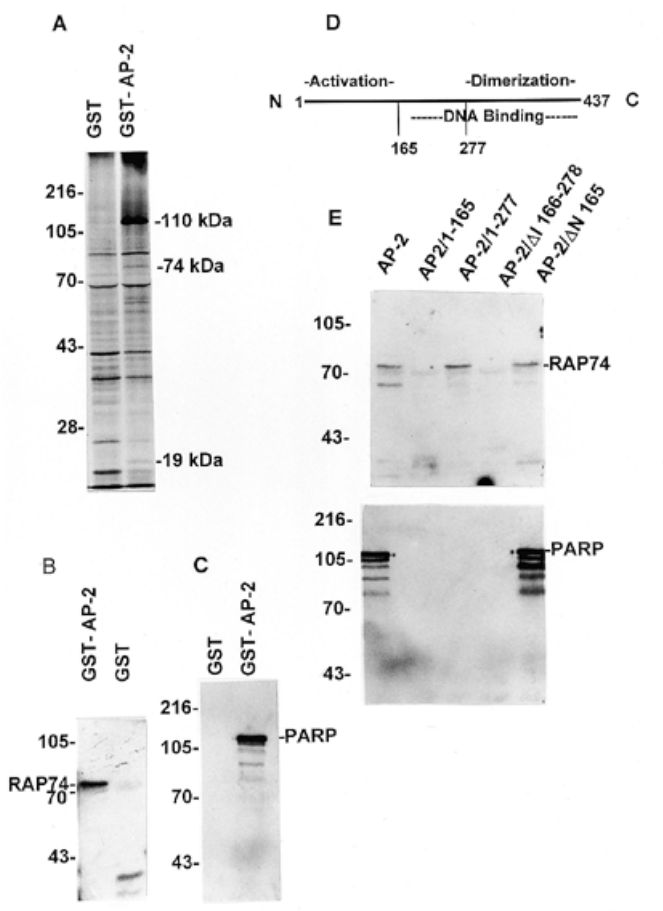

Figure 2. Analysis of AP-2-associated proteins. AP-2 protein binding assays were carried out with GST–AP-2 fusion protein and PA-1 cell nuclear extract as described in Materials and Methods. The molecular weight markers are shown on the left. (**A**) AP-2-interacting proteins. The three ^{35}S metabolically labeled polypeptides that specifically associated with the GST–AP-2 fusion protein are indicated. (**B**) Physical interaction of RAP74 with AP-2. A western blot containing the GST–AP-2-associated proteins was probed with a RAP74-specific rabbit polyclonal antiserum and the signals were visualized using the chemiluminescence technique with an anti-rabbit HRP antibody. (**C**) Physical interaction of PARP with AP-2. A western blot containing the GST–AP-2-associated proteins was probed with PARP-specific monoclonal antibody and the signals were visualized as above with an anti-mouse HRP antibody as described in Materials and Methods. (**D**) The functional domains of AP-2. The end points of different truncations of AP-2 at amino acids 165 and 277 are shown. (**E**) RAP74 interacts with the middle region and PARP interacts with the C-terminal region of AP-2. Experiments were carried out as described above in (B) and (C).

Mapping of the regions of AP-2 that interact with RAP74 and PARP

The regions of AP-2 that are necessary for RAP74 and PARP interaction were identified using immobilized GST–AP-2 fusion proteins containing various deletions in AP-2 sequences. The functional regions of AP-2 are shown in Figure 2D. The N-terminal one third of the molecule contains an activation domain (36). The DNA-binding domain is situated in the C-terminal two thirds of the molecule with an integral dimerization motif (37). The GST–AP-2/1–165 construct, which contains the

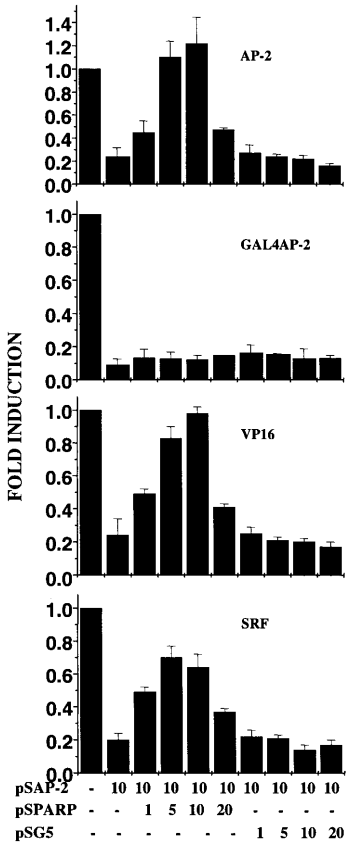

Figure 3. PARP relieves AP-2 transcriptional self-interference. Transient transfection experiments in the non-*ras* PA-1 subline and CAT assays were carried out as described in Materials and Methods. The amount of expression plasmids for AP-2 and PARP and the vector control pSG5 used for co-transfection are shown at the bottom. AP-2, GAL4–AP-2, VP16 and SRF activities were measured as described in the legend to Figure 1. The fold activity shown in each panel was calculated by measuring the percentage conversion of acetylated forms of [^{14}C]chloramphenicol and assuming the activity in the absence of AP-2 or PARP expression plasmid as 1.

amino acids 1–165, bound neither RAP74 nor PARP (Fig. 2E). GST–AP-2/1–277, which contains the amino acids between 1 and 277, interacted with RAP74 but did not bind PARP. GST–AP-2/ΔN 165, which contains the C-terminus of AP-2 from amino acid 166, specifically bound to RAP74 and PARP. The GST–AP-2/ΔI 166–278 construct, which was deleted for the internal amino acids between 166 and 278, failed to bind RAP74 or PARP, indicating that the amino acids between 166 and 278 of AP-2 are required for the interaction with these proteins. These experiments indicated that the C-terminal amino acids from 166 are necessary for interaction with PARP. This region of AP-2 contains its DNA-binding domain with an integral dimerization domain. RAP74 interacts with the central region of AP-2 between amino acids 166 and 278.

Relief of AP-2 transcriptional self-interference

The interaction of PARP and RAP74 with AP-2 suggested these proteins might play a role in AP-2-mediated transcriptional activity. A PARP expression plasmid under the control of an SV40 promoter was co-transfected into non-*ras* 9117 PA-1 cells with 10 μg pSAP-2, which is sufficient to induce self-interference. AP-2 transcriptional activity was measured using a 3× AP-2–CAT reporter plasmid. Co-transfection of an AP-2 expression plasmid with the AP-2 reporter plasmid resulted in ~4-fold inhibition of the endogenous AP-2 activity. Interestingly, co-transfection of PARP expression plasmid restored AP-2 transactivation activity in a dose-dependent manner (Fig. 3). Ten micrograms of pSPARP, the PARP expression plasmid, maximally elevated AP-2 activity 4- to 5-fold from the maximally inhibited level. Increasing the amount of PARP expression plasmid above this amount (i.e. 20 μg), however, did not effectively reverse AP-2 self-inhibition. No induction of AP-2 activity was seen when the parental vector pSG5 was transfected. pSG5 inhibited the AP-2 activity further by ~20%. The expression of AP-2 is driven by an SV40 promoter. An SV40 promoter-driven β-galactosidase expression vector pCH110 did not show a significant change in β-galactosidase enzyme activity when transfected with and without the PARP expression plasmid, indicating that PARP is not affecting the SV40 promoter and thereby altering AP-2 expression (data not shown). Western blot analysis showed no significant change in AP-2 expression when the nuclear extracts from the cell lines transfected with PARP expression plasmid as above were tested (data not shown). These experiments confirm that the change in AP-2 activity is not due to an alteration in AP-2 expression by PARP. We performed AP-2 *in vitro* transcription experiments to test whether PARP could restore AP-2 transcriptional self-interference *in vitro* as well. The effect of purified PARP protein on AP-2-mediated transcription was examined using HeLa cell nuclear extracts. Two AP-2-binding sites were cloned upstream of a c-*myc* minimal promoter linked to a 398 bp DNA sequence that lacks G residues. The absence of G residues enables degradation of non-specific transcripts using RNase T1 (29). The plasmid p052 which contains an unrelated *hsp70* heat shock promoter and a 298 bp DNA sequence with no G residues was used as an internal control in these *in vitro* transcription experiments. Figure 4 shows the transcription products of the two plasmids. The presence of AP-2 target sequences in the plasmid pcmyc-AP-2 enhanced the transcription many-fold compared with the parental plasmid pcmyc-PC, indicating the existence of endogenous AP-2 activity in HeLa cell nuclear extracts. Addition of recombinant AP-2 protein inhibited transcription from the pcmyc-AP-2 plasmid, indicating that AP-2 transcriptional self-interference occurs *in vitro* as well. When recombinant PARP protein was added with 200 ng of AP-2 protein the transcription from the pcmyc-AP-2 was restored in a dose-dependent manner. The transcriptional activity of pcmyc-AP-2 was maximally restored when 100 pg PARP protein was used in the assay. Transcription from the control plasmid p052 was not significantly altered in these experiments. An aliquot of 100 pg of PARP protein did not affect transcription from the parental control plasmid pcmyc-PC, indicating that the AP-2 sites in pcmyc-AP-2 are necessary for PARP-mediated restoration of transcription.

As shown in Figure 1, AP-2 also inhibits the activities of GAL4–AP-2, GAL4–VP16 and SRF. Co-transfection of pSPARP did not significantly restore the activity of GAL4–AP-2 that was inhibited by AP-2. This is consistent with our observation that PARP interacts with the C-terminal region of AP-2 that is lacking in the GAL4–AP-2 fusion protein which contains the N-terminal region amino acids 11–226. We have shown earlier that PC4, another coactivator that interacts with the N-terminus of AP-2, was able to reduce AP-2 cross-interference

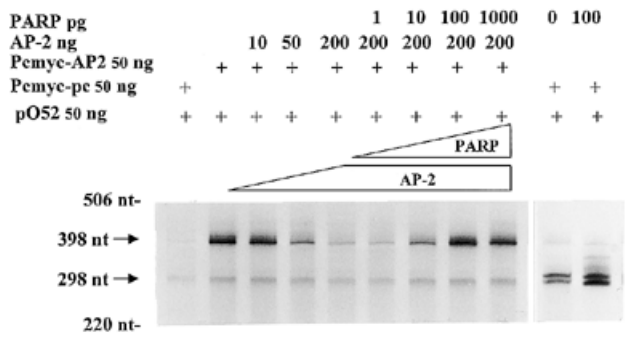

Figure 4. PARP relieves AP-2 transcriptional self-interference *in vitro*. *In vitro* transcription reactions using HeLa cell nuclear extracts were performed as described in Materials and Methods. The template plasmids used in each assay are indicated on top. The amount of recombinant AP-2 protein or recombinant PARP protein added to the *in vitro* transcription reaction is shown. A 398 nt transcription product from pcmyc-AP-2 or pcmyc-PC and a 298 nt transcription product from the control plasmid p052 are shown on the left. The end-labeled nucleotide mobility markers are marked on the left.

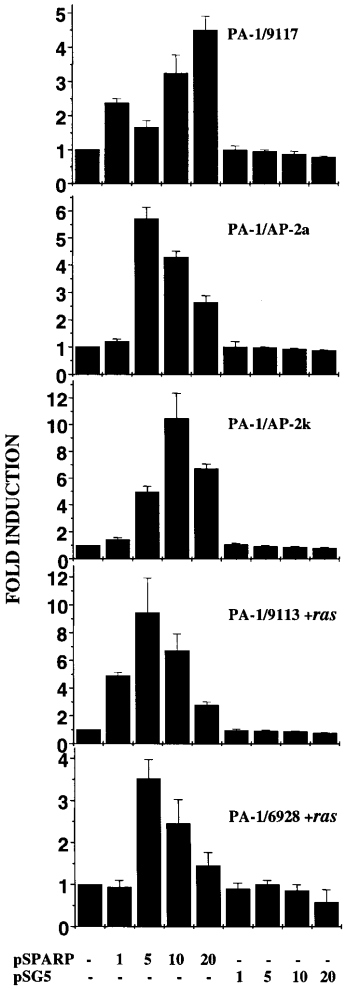

Figure 5. PARP restores AP-2 activity in *ras*-transformed cell lines and AP-2 overexpressor cell lines. Transient transfection experiments and CAT assays were carried out as described in Materials and Methods. The amount of expression plasmid PARP and the vector control pSG5 used for co-transfection are shown at the bottom. The cell lines transfected were 9117, a non-*ras* PA-1 subline, the AP-2 overexpressor cell lines PA-1/AP-2a and PA-1/AP-2b, 9113, an N-*ras* G12D mutant PA-1 cell line, and 6928, a *ras*-transfected PA-1 subline. The fold activity shown in each panel was calculated by measuring the percentage conversion of acetylated forms of [^{14}C]chloramphenicol and assuming the activity in the absence of PARP expression plasmid as 1.

with GAL4–AP-2 (4). These results suggested that during AP-2 overexpression the protein sequesters more than one coactivator. PARP was able to abolish the cross-interference of AP-2 with VP16 and significantly reduced the cross-interference with SRF. In the above experiments the control expression vector pSG5 did not significantly affect AP-2 cross-interference with GAL4–AP-2, VP16 and SRF. These experiments indicate that PARP is a transcriptional coactivator that is titrated away during AP-2 overexpression. The reversal of AP-2 cross-interference of VP16 and SRF suggested that PARP is also used as a coactivator by the activators VP16 and SRF.

In similar experiments RAP74 neither significantly affected endogenous AP-2 activity in PA-1 cells nor relieved AP-2 transcriptional self-interference (not shown). Transfection of RAP74 expression plasmid pCGNRAP74 into N-*ras*-transformed PA-1 cells also did not increase AP-2 transactivation activity (not shown). The combination of Rap74 and Rap30, the two subunits of transcription factor TFIIF, also did not affect AP-2 activity (not shown). These experiments suggest that the RAP74 subunit of TFIIF is not a coactivator for AP-2-mediated transcription and it is not a significant factor sequestered by overexpression of AP-2 causing transcription self-interference.

We have characterized three different factors, RAP74, PARP and PC4 (4) that interact with AP-2. PARP and PC4 modulate AP-2-mediated transcription and relieve AP-2 transcriptional self-interference. We tested whether the combination of these factors may enhance the AP-2 activity further and relieve AP-2 transcriptional self-interference more efficiently. In co-transfection experiments RAP74 failed to influence AP-2 activity in the presence or absence of PARP or PC4. In similar experiments PARP and PC4 together increased AP-2 activity to the same level activated by PARP alone and relieved AP-2 transcriptional self-interference about the same level as they did independently (results not shown). These studies show that PARP and PC4 are independent coactivators for AP-2-mediated transcription and do not affect AP-2 activity synergistically.

PARP relieves AP-2 transcriptional self-interference in AP-2- and *ras*-transformed cells

The endogenous AP-2 activity was elevated significantly in a dose-dependent manner when pSPARP was transfected into PA-1 cells (Fig. 5). Purified recombinant PARP protein restored the AP-2 activity inhibited by high levels of AP-2 protein in *in vitro* transcription assays using HeLa cell nuclear extracts (Fig. 4). These results strongly indicated that PARP is a coactivator for AP-2-mediated transcription. We have previously shown that AP-2 transcriptional self-interference results in reduced AP-2 activity which results in *ras* oncogene-induced transformation of PA-1 cells (3). The *ras* oncogene induces the expression of AP-2 mRNA and increases the level of AP-2 protein. However, little AP-2 transactivation activity is seen in these cells. Similarly,

derivatives of PA-1 cell lines that constitutively overexpress AP-2, PA1/AP-2a and PA-1/AP-2k have tumorigenic properties similar to *ras*-transformed cell lines with low AP-2 activity. The PARP expression plasmid was transfected into AP-2-overexpressing PA-1 sublines PA1/AP-2a, PA-1/AP-2k and *ras*-transformed PA-1 cell lines 9113, which contains a spontaneously activated *ras* oncogene, and 6928, which contains a transfected *ras* oncogene. PARP significantly enhanced the AP-2 transactivation activity in all these tumorigenic cells (Fig. 5). Optimal induction of AP-2 activity was seen in a window of PARP concentration with a maximal increase of >5-fold in PA-1/AP-2a, >10-fold in PA-1/AP-2k, >9-fold in 9113 and >3-fold in 6928 cell lines. As PARP restores AP-2 activity in *ras*-transformed cells and in cell lines that constitutively overexpress AP-2, PARP is a potential candidate that can suppress transformation caused by the *ras* oncogene and AP-2.

PARP interacts with additional members of the AP-2 family transcription factors

AP-2 activates transcription from the c-*erbB*2 proto-oncogene in human mammary carcinoma (33). Preliminary evidence suggests that AP-2 and PARP proteins strongly associate with each other in these cells. We selected the cell line MDA-MB453, that overexpresses AP-2, and tested whether AP-2 interacts with PARP in this cell line. Nuclear extracts were subjected to co-immunoprecipitation studies using antibodies to AP-2 to test for *in vivo* association of PARP with AP-2. As shown in Figure 6A, antibody to PARP recognized a strong 110 kDa signal in the AP-2 immunoprecipitation complex. A similar co-immunoprecipitation of AP-2 and PARP was seen in PA-1 teratocarcinoma cells (not shown). These studies indicate that AP-2 and PARP interact in breast cancer cell line MDA-MB453. The key role played by transcription factor AP-2 in embryonal development and differentiation and e-*erbB*2-overexpressing mammary carcinomas led to the identification of AP-2β and AP-2γ transcription factors in murine and human systems (34,38). These members of the AP-2 family show strong conservation of amino acid sequence at the C-termini in their DNA-binding and dimerization domains. The amino acid sequence of the DNA-binding domain of human AP-2 (now called AP-2α) is 86% identical to that of AP-2β and 79% to that of AP-2γ. The conservation is less at the N-termini in their activation domains. Since PARP interacts with the C-terminal region of AP-2α we tested whether PARP could interact with AP-2β and AP-2γ. Murine AP-2β and a human AP-2γ were *in vitro* synthesized and then mixed with *in vitro* synthesized PARP. Human and murine AP-2β sequences differ only by a single amino acid at position 425. Antibody against each one of the proteins was used to co-immunoprecipitate the other protein. A PARP antibody was able to co-immunoprecipitate AP-2α, AP-2β and AP-2γ proteins (Fig. 6B). Similarly, antibodies to AP-2α, AP-2β or AP-2γ co-immunoprecipitated PARP. These results indicate that PARP interacts with all the three forms of AP-2 and demonstrates that the interaction region of PARP is at the C-terminal end of AP-2.

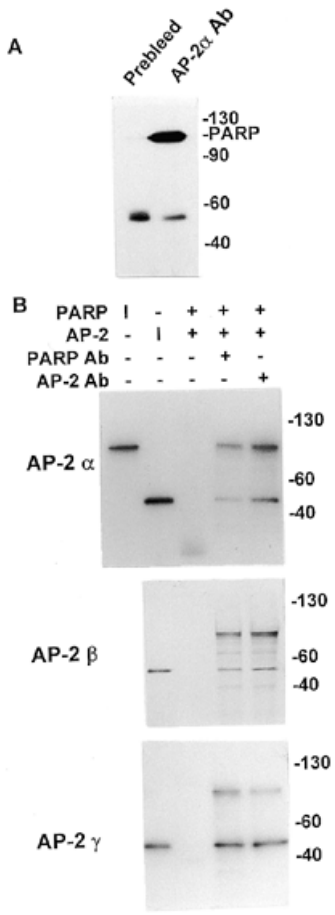

Figure 6. PARP interacts with the AP-2 family of transcription factors. The molecular weight markers are shown on the right. (**A**) AP-2 and PARP associate in the MDA-MB453 mammary carcinoma cell line. Immunoprecipitation experiments using the nuclear extract of cell line MDA-MB453 and AP-2α antibody were carried out, the samples resolved by 10% SDS–PAGE, blotted onto a nitrocellulose membrane and probed with a PARP-specific antibody as described in Materials and Methods. (**B**) PARP interacts with AP-2α, AP-2β and AP-2γ. PARP, AP-2α, AP-2β and AP-2γ proteins were synthesized *in vitro* and co-immunoprecipitation studies using a monoclonal antibody specific for PARP or a monoclonal antibody specific for both AP-2α and AP-2β or a polyclonal antibody for AP-2γ were carried out as described in Materials and Methods. I refers to one-sixth amount of input protein added without immunoprecipitation. The samples were resolved by 14% SDS–PAGE.

DISCUSSION

We have previously shown that the *ras* oncogene induces a high level of the mRNA for transcription factor AP-2. Overexpression of AP-2 causes transcriptional self-interference and this process leads to tumorigenicity in the human teratocarcinoma cell line PA-1. Because overexpression of AP-2 inhibits the activities of GAL4–AP-2 fusion protein we concluded that transcriptional interference of AP-2 occurs independently of sequence-specific DNA binding. These data indicated that sequestration of co-activators is the likely mechanism for self-interference. These initial observations were supported by experiments in which AP-2 inhibited the activities of transcription factors with unrelated target DNA specificity such as GAL4–VP16 and SRF. We hypothesized that these putative coactivators were necessary for the normal functioning of other activators that are involved in cell growth control. Limiting their availability to other transcriptional activators impaired their activity and promoted a pleiotropic signal for abnormal cell growth. An extension of this hypothesis is that

regulating the level of the limiting coactivators within a cell should restore cell growth control. To test this hypothesis we searched for the coactivators utilized by AP-2 as a transcriptional activator. We identified three proteins (19, 74 and 110 kDa) that physically interacted with GST–AP-2 fusion protein and characterized their role in AP-2-transcriptional activation. The 19 kDa polypeptide was identified earlier as the positive coactivator PC4 (4).

In this report we demonstrate that the 110 kDa protein is the enzyme PARP. PARP interacts with the C-terminus of AP-2 where a helix–span–helix dimerization motif is situated (37). PARP binds a number of cellular proteins and has been shown to have a protein- and self-association region at the N-terminus ending at amino acid 606 (39). The N-terminal 450 amino acids have been shown to be sufficient for the coactivator function of PARP in *in vitro* transcription assays (15). These regions of AP-2 and PARP do not reveal any interaction motif common to both. Further experiments will be necessary to determine the mechanism by which these molecules interact. The N-terminus of AP-2, which contains its activation domain, fused to a GAL4 DNA-binding domain is capable of inducing self-interference and transformation of PA-1 cells (3). This suggested that the coactivator that could relieve AP-2 self-interference should be interacting with the N-terminus of AP-2. However, PARP enhances AP-2 transcriptional activity and relieves AP-2 transcriptional self-interference in PA-1 cells. The reversal of AP-2 self- and cross-interference is effective in a window of PARP concentration where the optimal concentration restores the transcriptional activity but lower or higher concentrations are less effective. Low concentrations of PARP presumably are not sufficient for relief of self-interference. PARP may require certain modification for coactivator function, which may not be efficient, when PARP is at high levels. In our studies, purified recombinant PARP protein enhances AP-2 activity in *in vitro* transcription assays using HeLa cell nuclear extracts. Our results confirm that PARP is a transcriptional coactivator for AP-2-mediated transcription and that AP-2 sequesters more than one coactivator during its overexpression. Different domains of AP-2 interact with each coactivator. PARP interacts with the C-terminus of AP-2 while PC4 interacts with the N-terminus (4). Interestingly, a cumulative enhancement of AP-2 activity was not seen when PARP and PC4 were transfected together. Possibly AP-2 substitutes coactivators without depending on any one coactivator. Alternatively, binding of PARP or PC4 may induce AP-2 active conformational changes that would prevent it from interacting with another coactivator. The GAL4–AP-2 fusion protein that contains the N-terminal sequences of AP-2 is responsive to PC4 (4), indicating that AP-2 retains the PC4-responsive conformation while deleted for PARP-responsive sequences. *In vitro* experiments that measure the stoichiometry of the coactivator interactions with AP-2 are necessary to fully understand how coactivators participate in AP-2-mediated transcription.

Investigations by other laboratories have noted the influence of PARP on transcription. PARP was shown to interact with transcription factor YY1 and alter RNA polymerase II-dependent transcription (40,41). PARP binds transcription factor p53 and alters its DNA-binding properties and thereby affects its transcriptional activity (11,42). The transcriptional coactivator PC1 that enhances GAL4-AH and NFκB TA1 activator-dependent transcription *in vitro* was identified as PARP (15). PARP reactivates AP-2 activity in *ras* oncogene-transformed PA-1 cells which have abundant AP-2 mRNA and protein. This suggests that PARP may be able to suppress transformation induced by AP-2 and *ras* oncogene. In our previous PA-1 somatic cell hybridization studies the loss of *ras* oncogene tumor suppressor activity was found to be due to the loss of chromosomes 1, 4 and 11 (43). It is interesting to note that the PARP gene is located on chromosome 1 (44). Several studies have observed suppression of *ras* oncogene-mediated tumorigenicity by PARP (45–47). 5-Iodo-6-aminobenzopyrone, a non-covalent binding ligand of PARP, reverses *ras* oncogene-transformation when administered in rat (46), in E-*ras*-transformed cultured cell lines and prostatic carcinoma cells (10). The mechanism by which PARP reverts malignancy is not clear and is thought to be due to the colligative properties of PARP with other molecules (7). We are currently investigating the tumor suppressor properties associated with the coactivator function of PARP. Transcription factors AP-2α and AP-2γ up-regulate c-*erbB2* gene expression in breast cancer cell lines (34). The association of PARP with AP-2α and AP-2γ indicates that PARP may play a role in c-*erbB2* overexpression and mammary carcinoma.

The RAP74 subunit of transcription factor TFIIF has been shown to relieve SRF and VP16 self-interference in *in vitro* studies (17,18). RAP74 interacts with the central region of AP-2 that contains a portion of its DNA-binding domain. Our studies indicate that RAP74 fails to influence AP-2-mediated transcription either alone or with RAP30, the other subunit of TFIIF. These observations suggest that RAP74 is not a coactivator for AP-2-mediated transcription and it is not the factor that was sequestered by overexpression of AP-2 causing transcriptional self-interference. The functional significance of the interaction of RAP74 with AP-2 remains to be determined.

Transcriptional self- and/or cross-interference have been observed with many transcriptional activators, including GAL4, GCN4, VP16, E1A, TEF-1, SRF, p53, E2F-1 and steroid and hormone receptors (48–53). The mechanism by which a transcriptional activator affects its own activity or the activities of other activators is not clearly understood. Our studies provide a mechanistic model for transcriptional self- and cross-interference involving transcriptional coactivators. Since the coactivators are shared by many activators our model strongly implicates the transcriptional self- and cross-interference as key regulators of many cellular functions, including cellular transformation. If transformation of PA-1 cells is due to sequestration of common coactivator(s) by AP-2, then overexpression of any of the activators cross-interfered by AP-2 overexpression also might cause transformation of PA-1. This possibility was tested with the activator GAL4–VP16 by establishing stable cell lines of clone 1 PA-1 cells that constitutively express GAL4–VP16 from an SV40 promoter. When a pool of GAL4–VP16-transfected colonies of clone 1 PA-1 cells was injected into nude mice, they induced tumors equivalent to N-*ras* PA-1 cells and AP-2 overexpressor cell lines. These studies suggest that sequestration of a coactivator by overexpression of AP-2 (or VP16) confers tumorigenicity to PA-1 cells and indicate that the coactivators are potential tools that can be exploited to suppress transformation.

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REFERENCES

1 Calkhoven,C.F. and Ab,G. (1996) *Biochem. J.*, **317**, 329–342.
2 Hill,C.S. and Treisman,R. (1995) *Cell*, **80**, 199–211.
3 Kannan,P., Buettner,R., Chiao,P.J., Yim,S.O., Sarkiss,M. and Tainsky,M.A. (1994) *Genes Dev.*, **8**, 1258–1269.
4 Kannan,P. and Tainsky,M.A. (1999) *Mol. Cell. Biol.*, **19**, 899–908.
5 de Murcia,G., Schreiber,V., Molinete,M., Saulier,B., Poch,O., Masson,M., Niedergang,C. and Menissier,d.M. (1994) *Mol. Cell. Biochem.*, **138**, 15–24.
6 Lindahl,T., Satoh,M.S., Poirier,G.G. and Klungland,A. (1995) *Trends Biochem. Sci.*, **20**, 405–411.
7 Kun,E. (1998) *Int. J. Mol. Med.*, **2**, 131–142.
8 Simbulan-Rosenthal,C.M., Rosenthal,D.S., Boulares,A.H., Hickey,R.J., Malkas,L.H., Coll,J.M. and Smulson,M.E. (1998) *Biochemistry*, **37**, 9363–9370.
9 Cole,G.A., Bauer,G., Kirsten,E., Mendeleyev,J., Bauer,P.I., Buki,K.G., Hakam,A. and Kun,E. (1991) *Biochem. Biophys. Res. Commun.*, **180**, 504–514.
10 Bauer,P.I., Kirsten,E., Varadi,G., Young,L.J., Hakam,A., Comstock,J.A. and Kun,E. (1995) *Biochimie*, **77**, 374–377.
11 Vaziri,H., West,M.D., Allsopp,R.C., Davison,T.S., Wu,Y.S., Arrowsmith,C.H., Poirier,G.G. and Benchimol,S. (1997) *EMBO J.*, **16**, 6018–6033.
12 Masutani,M., Nozaki,T., Wakabayashi,K. and Sugimura,T. (1995) *Biochimie*, **77**, 462–465.
13 Duriez,P.J. and Shah,G.M. (1997) *Biochem. Cell Biol.*, **75**, 337–349.
14 Slattery,E., Dignam,J.D., Matsui,T. and Roeder,R.G. (1983) *J. Biol. Chem.*, **258**, 5955–5959.
15 Meisterernst,M., Stelzer,G. and Roeder,R.G. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 2261–2265.
16 Zawel,L. and Reinberg,D. (1992) *Curr. Opin. Cell Biol.*, **4**, 488–495.
17 Zhu,H., Joliot,V. and Prywes,R. (1994) *J. Biol. Chem.*, **269**, 3489–3497.
18 Joliot,V., Demma,M. and Prywes,R. (1995) *Nature*, **373**, 632–635.
19 Zeuthen,J., Norgaard,J.O., Avner,P., Fellous,M., Wartiovaara,J., Vaheri,A., Rosen,A. and Giovanella,B.C. (1980) *Int. J. Cancer*, **25**, 19–32.
20 Tainsky,M.A., Krizman,D.B., Chiao,P.J., Yim,S.O. and Giovanella,B.C. (1988) *Anticancer Res.*, **8**, 899–913.
21 Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
22 Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.
23 Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
24 Buettner,R., Kannan,P., Imhof,A., Bauer,R., Yim,S.O., Glockshuber,R., Van Dyke,M.W. and Tainsky,M.A. (1993) *Mol. Cell. Biol.*, **13**, 4174–4185.
25 Graham,F.L. and Eb,A.J. (1973) *Virology*, **52**, 456–467.
26 Luckow,B. and Schutz,G. (1987) *Nucleic Acids Res.*, **15**, 5490.
27 Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
28 Miller,J.H. and Schmeissner,U. (1979) *J. Mol. Biol.*, **131**, 223–248.
29 Sawadogo,M. and Roeder,R.G. (1985) *Cell*, **43**, 165–175.
30 Sallmann,F.R., Plancke,Y.D. and Poirier,G.G. (1998) *Mol. Cell. Biochem.*, **185**, 199–203.
31 Wang,J.C. and Van Dyke,M.W. (1994) *Biochim. Biophys. Acta*, **1218**, 308–314.
32 Treisman,R. (1985) *Cell*, **42**, 889–902.
33 Bosher,J.M., Williams,T. and Hurst,H.C. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 744–747.
34 Bosher,J.M., Totty,N.F., Hsuan,J.J., Williams,T. and Hurst,H.C. (1996) *Oncogene*, **13**, 1701–1707.
35 de Murcia,G. and Menissier,d.M. (1994) *Trends Biochem. Sci.*, **19**, 172–176.
36 Williams,T. and Tjian,R. (1991) *Genes Dev.*, **5**, 670–682.
37 Williams,T. and Tjian,R. (1991) *Science*, **251**, 1067–1071.
38 Moser,M., Imhof,A., Pscherer,A., Bauer,R., Amselgruber,W., Sinowatz,F., Hofstadter,F., Schule,R. and Buettner,R. (1995) *Development*, **121**, 2779–2788.
39 Buki,K.G., Bauer,P.I., Hakam,A. and Kun,E. (1995) *J. Biol. Chem.*, **270**, 3370–3377.
40 Oei,S.L., Griesenbeck,J., Schweiger,M., Babich,V., Kropotov,A. and Tomilin,N. (1997) *Biochem. Biophys. Res. Commun.*, **240**, 108–111.
41 Oei,S.L., Griesenbeck,J., Ziegler,M. and Schweiger,M. (1998) *Biochemistry*, **37**, 1465–1469.
42 Malanga,M., Pleschke,J.M., Kleczkowska,H.E. and Althaus,F.R. (1998) *J. Biol. Chem.*, **273**, 11839–11843.
43 Krizman,D.B., Giovanella,B.C. and Tainsky,M.A. (1990) *Somat. Cell Mol. Genet.*, **16**, 15–27.
44 Cherney,B.W., McBride,O.W., Chen,D.F., Alkhatib,H., Bhatia,K., Hensley,P. and Smulson,M.E. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 8370–8374.
45 Nagao,M., Nakayasu,M., Aonuma,S., Shima,H. and Sugimura,T. (1991) *Environ. Health Perspect.*, **93**, 169–174.
46 Tseng,A.J., Lee,W.M., Jakobovits,E.B., Kirsten,E., Hakam,A., McLick,J., Buki,K. and Kun,E. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 1107–1111.
47 Troll,W., Garte,S. and Frenkel,K. (1990) *Basic Life Sci.*, **52**, 225–232.
48 Gill,G. and Ptashne,M. (1988) *Nature*, **334**, 721–724.
49 Magae,J., Illenye,S., Tejima,T., Chang,Y.C., Mitsui,Y., Tanaka,K., Omura,S. and Heintz,N.H. (1997) *Oncogene*, **15**, 759–769.
50 Shibata,H., Spencer,T.E., Onate,S.A., Jenster,G., Tsai,S.Y., Tsai,M.J. and O'Malley,B.W. (1997) *Recent Prog. Hormone Res.*, **52**, 141–164.
51 Tavernarakis,N. and Thireos,G. (1995) *Mol. Gen. Genet.*, **247**, 571–578.
52 Brockmann,D., Feng,L., Kroner,G., Tries,B. and Esche,H. (1994) *Virology*, **198**, 717–723.
53 Xiao,J.H., Davidson,I., Matthes,H., Garnier,J.M. and Chambon,P. (1991) *Cell*, **65**, 551–568.