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Saccharomyces cerevisiae multifunctional protein RAP1 binds to a conserved sequence in the Polyoma virus enhancer and is responsible for its transcriptional activity in yeast cells

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The Polyoma virus enhancer (A + B domain) activates transcription in *Saccharomyces cerevisiae* when joined to appropriate yeast promoter elements. We demonstrate by DNase I footprints and inhibition of binding by specific antibody, that the yeast protein RAP1 binds to the B-domain of the Polyoma enhancer and, at least in some promoter contexts, is responsible for transcriptional activity of the enhancer B-domain in yeast Close matches to a consensus RAP1-binding site are also present in other viral enhancers.

Transcription factor; Polyoma enhancer; *Saccharomyces cerevisiae*; RAP1

1. INTRODUCTION

Viral transcriptional enhancers consist of multiple sequences that match DNA binding activator proteins present in host tissues [1,2]. In a previous work [3,4] we replaced a native UAS element in a truncated yeast promoter with the Polyoma virus enhancer and observed activation of transcription of a reporter gene. This result suggests that the enhancer element contains evolutionarily conserved binding sites for one or more yeast proteins, which can drive RNA synthesis in concert with the basal transcriptional machinery in yeast.

We have screened for such yeast proteins that bind to the Polyoma enhancer and identify a yeast protein that binds in vitro to a sequence within the B-domain, one of the two major domains of the Polyoma enhancer. We show that this protein is repressor/activator protein RAP1 (also known as TUF1/GRF1) [5] and that, in some promoter contexts, it is by itself responsible for enhancer activity in yeast. RAP1 is a scaffold-associated structural protein that plays a multifunctional role in the cell: activation and silencing of transcription as well as chromosome maintenance and stimulation of meiotic recombination [5–7]. Although such a protein has so far not been described in other organisms besides yeast, the presence of potential RAP1 binding sites in Polyoma and other viral enhancers is suggestive of the presence of a RAP1 protein homologue in mammals.

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2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

In *E. coli*, plasmids were usually transformed and maintained in the rec A strain HB101 (F^-, hsd, S20, rB mB, recA13, ara-14, proA2, lacY1, galK2, rpsL20 Smr, xyl5, mtl-1, supE-44), selecting for ampicillin resistance For preparing DNA to be cut with *Stu*I endonuclease, plasmids were transformed into *E. coli* strain 577 dcm^-.

In *Saccharomyces cerevisiae*, plasmids were usually transformed and maintained in yeast strain ScKY117 (a, ura3–52, trp1-del1, his3-del200, ade2–101, lys2–801).

Protein extract was prepared from the diploid strain DE 417 (a etrp1-del1, his3-del200, ade2–101, lys2–801, trp1–289).

To examine RAP1 dependence of transcription we constructed the following strain: (his3-del200, ade2–101, trp1, ura3–52, $gnc1^{ts}$); $gnc1^{ts}$ is a temperature-sensitive mutation in gene RAP1 (a gift from M. Breitenbach). Standard genetic techniques were used to integrate the test plasmids at the TRP1 locus on chromosome IV. Copy number and site of integration were determined by Southern blotting of appropriate restriction enzyme digests of yeast DNA.

Test plasmids have been described previously [3] In particular, pGM3181 carries TRP1 as a selectable marker and the test gene HIS3 with an *Eco*RI linker at nt −80, while all sequences upstream of this site, including the UAS, are deleted.

2.2. *DNA binding assay, immunoassay and protease clipping*

Binding reactions, using 5–10 μg of total protein extract were carried out as described [8] with the following modifications: total volume of the binding reaction was 20 μl, $1–5 \times 10^4$ cpm of probe and 1 μg of poly(dI-dC) was added to the reaction as non-specific competitor; binding reactions were carried out for 10 min at 20°C loaded directly on 5% polyacrilamide gels and run in 0.5 × TBE at 200 V, 25 mA. Competition experiments were carried out by adding unlabelled competitor to the reaction 5 min before adding the probe.

For immunoassay, 5 μg of crude extracts were incubated for 30 min on ice with varying amounts of either polyclonal antibody raised against RAP1 (a gift from S Gasser) or of preimmune serum, in binding buffer and poly(dI-dC), as described above. Probe was then added and the binding assay was carried out as usual.

Limited proteolysis of complexes was performed by incubating sam-

ples after the binding reaction for 10 min at room temperature in the presence of different amounts of proteinase K (10 pg/μl; 0.1 ng/μl; 1 ng/μl). Samples were then directly electrophoresed on 5% polyacrylamide gels in 0.5 × TBE.

2.3. *Preparation of protein extracts*

Crude yeast extracts were prepared as described [9].

2.4. *Preparation of probes, footprinting assays*

The Polyoma enhancer B-domain fragment was cloned by *Bam*HI linkers into vector pGEM 4; the plasmid was first cut at the *Xba*I site, labelled on the coding strand with [α^{32} P]dCTP with Klenow enzyme and recut with *Sma*I. The labelled B-domain fragment was purified on a 5% polyacrylammide gel and used for footprinting. Binding reactions containing 1–3 × 10^5 cpm of probe and either 20–30 μg of protein extract or 100 ng of purified RAP1 were carried out for 10 min at 20°C. $MgCl_2$ 10 mM final and DNase I (10–20 ng/μl final) were added to the mixture and digestion was carried out for 1 min at 25°C; the reaction was stopped by the addition of 25 mM final EDTA and samples were immediately loaded on a non-denaturing polyacrylamide gel; the complexed and free probes were cut out from the gel, electroeluted, phenol extracted and run on a 6% polyacrylamide, 8 M urea sequencing gel. A Maxam and Gilbert A+G reaction was carried out on the same probe to localize position of the footprint.

2.5. *Purification of RAP1 produced in* E. coli

E coli strain BL 21 [10] was used to induce expression of RAP1 protein which was then purified by preparative SDS-PAGE according to Hager et al. [11] except that 8 M urea was used as denaturing agent.

2.6. *RNA preparation and Northern blotting*

Cells were grown in supplemented minimal medium lacking tryptophan. Isogenic rap^{ts} and RAP wt strains were grown at 26°C from spores dissected from the same ascus. Aliquots of the cultures were incubated for 4 h at 37°C or at 26°C and RNA was extracted as described [3].

3. RESULTS

3.1. *Yeast proteins that bind to the Polyoma enhancer*

The Polyoma enhancer (as other viral enhancers) is a functionally and structurally composite control element; it is contained within a segment of 246 bp (nt 5022–5267) essential for viral transcription and replication in differentiated mouse cells (Fig. 1A) [2].

Chromatography of total yeast extracts on heparin-Sepharose fractionates two classes of proteins that bind specifically to the enhancer in gel-retardation assays: one, eluting at a concentration of 0.3 M KCl, binds to the B-domain and one that elutes at 0.6 M KCl and binds to the A-domain (data not shown) (see scheme in Fig. 1A). The protein that binds to the latter domain

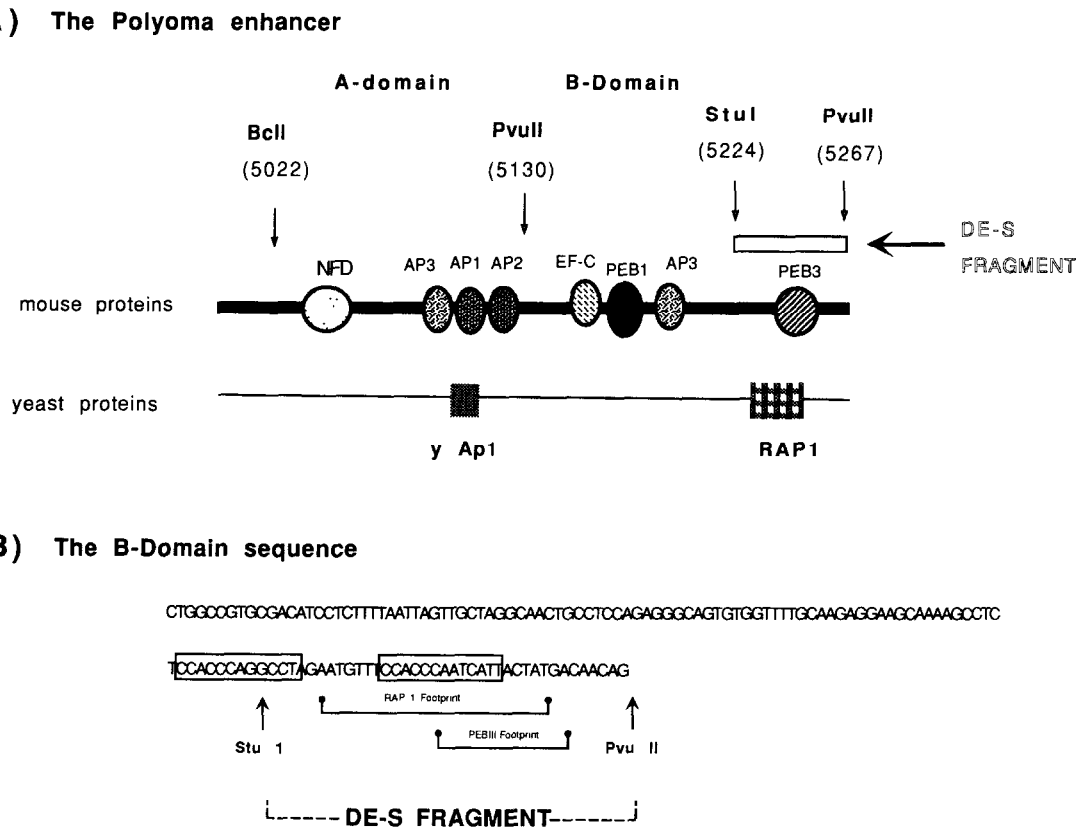

Fig. 1. (A) The Polyoma enhancer [31]. Two major functional domains have been identified within the Polyoma enhancer: the A-domain (109 bp) located between the *Bcl*I (nt 5022) and *Pvu*II (nt 5130) restriction sites, and the B-domain (137 bp) located between the *Pvu*II (nt 5130) and the *Pvu*II (nt 5267) restriction sites [2]. Shown in the figure are approximate locations of binding sites for mouse [31] and yeast (this paper) nuclear proteins. (B) B-Domain sequence. The DE-S fragment is indicated (between *Stu*I and *Pvu*II restriction sites). Boxed regions indicate the two RAP1 consensus sequences. Also indicated is the RAP1 footprint (yeast) and the so-called Peb III footprint (mammals; see section 4). The DE-S fragment is the minimal region necessary for protein binding to the B-domain in yeast.

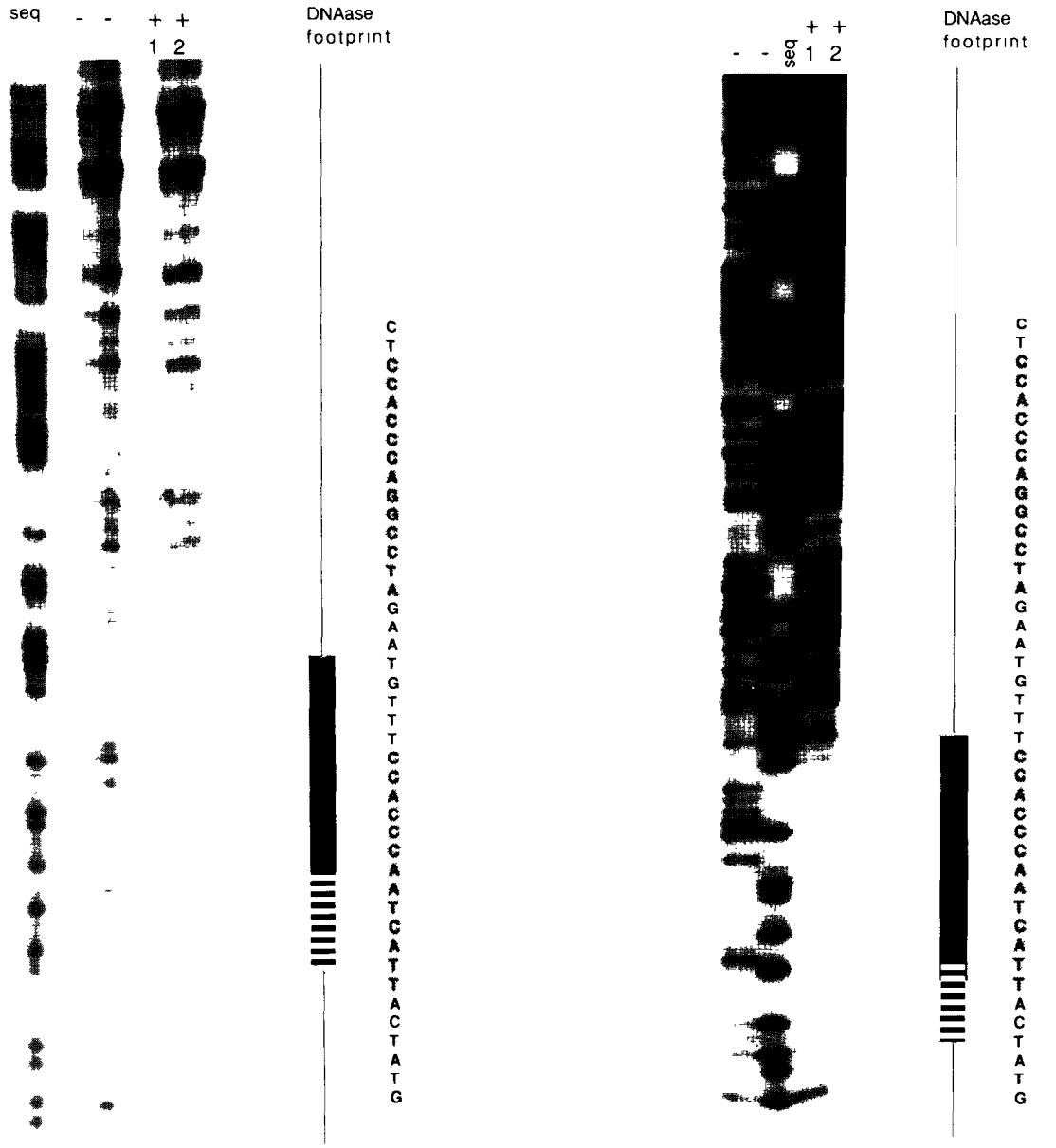

Fig. 2. DNase I protection on the B-domain. DNAse I protection conferred by incubation of end-labelled B-domain fragment with either total yeast extract (left panel) or purified RAP1 protein (right panel) (for details see section 2.4). Lanes '−' indicate non-complexed probe; lanes '+' indicate complexed probe; lane 'seq' contains an A+G Maxam and Gilbert reaction Duplicate lanes contain 100 μg/ml and 200 μg/ml of DNase I

appears, by its sequence specificity, to be a member of the yAP1 family (unpublished results); members of this family have been shown to bind to the SV40 enhancer [12,13].

We looked more carefully at the B-domain, and identified the region responsible for protein binding by deletion mapping of this domain (nt 5224 and 5267, the DE-S fragment; Fig. 1A and B) (data not shown).

DNase I footprinting of the B-domain was carried out on the 137 bp *Pvu*II fragment located between nt 5130–5267. 24 bp are protected and contain the sequence CCACCCAATCATT (Fig. 1B, boxed region and Fig. 2, left panel); this sequence diverges by only two nucleotides from the consensus binding site for yeast factor RAP1 [14].

Another adjacent potential RAP1-binding site is destroyed by the *Stu*I cut used to create the DE-S fragment in the deletion mapping experiments (Fig. 1B). Actually, an oligonucleotide containing this site in isolation binds RAP1 very poorly in vitro (data not shown) indicating that the sequence context of the RAP1 consensus is also important.

The same DNase I footprint of the B-domain was observed when this DNA segment was reacted with purified RAP1 (prepared after expression of the cloned gene in *E. coli*;) (Fig. 2, right panel).

3.2. *Identification of the yeast protein that binds to the B-domain*

We verified the hypothesis that the yeast protein bound to the B-domain was RAP1 by immunoassay. Pre-incubating *S. cerevisiae* extracts with polyclonal antibodies raised against RAP1 before the binding reaction alters the mobility of the complex on DE-S fragment. Pre-incubation with preimmune serum has no effect (Fig. 3). This is further and compelling evidence that, under our experimental conditions, RAP1 is involved in the formation of the protein–DNA complex on the B-domain.

3.3. *Only RAP1 binds to the Polyoma enhancer B-domain*

In yeast promoters, RAP1 is found to activate transcription in conjunction with other UAS elements [6,15,16]. We could not detect other abundant proteins binding to the B-domain in vitro, although less abundant transcriptional activators might go undetected in the mobility shift assay. RAP1 has been shown in some cases to exert its effects by interaction with other proteins. In some contexts its transcriptional activity is mediated by GAL11 [17] while transcriptional repression, upon binding to silencers, and telomere length (and chromosome maintenance), upon binding to telomeres, is affected in association with yet another protein (RIF) [18].

We have tested for the presence of other proteins bound to the B-domain fragment by comparing the products of limited proteolytic cleavage of DNA–protein complexes formed with crude extract and with purified RAP1. The patterns of partial degradation are identical, suggesting that the only protein in the extracts binding to the enhancer is RAP1 (Fig. 4).

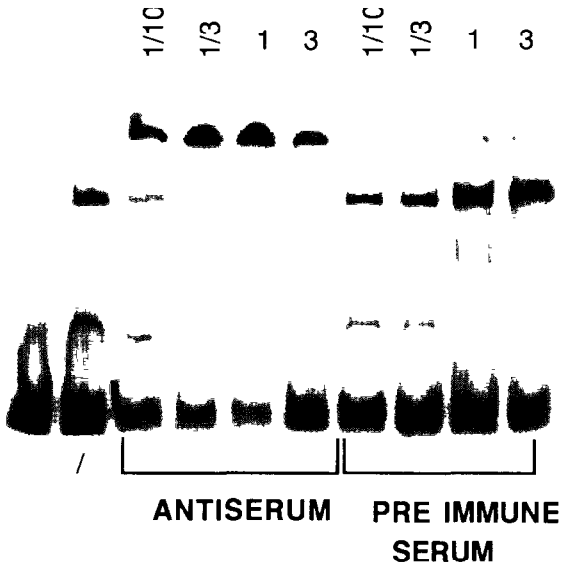

Fig. 3. Effect of antiserum against RAP1 on complex formation after incubation of 5 μg of total yeast extract with labelled DE-S fragment from the Polyoma enhancer.

Fig. 4. Effects of limited proteolysis on electrophoretic mobility shifts of protein/B-domain complexes: comparison of proteinase K clipping patterns obtained after incubation of either 5 μg of crude yeast extract or 100 ng of purified RAP 1 protein to the B-domain fragment. Lanes '/' indicate no proteinase K in the reaction

3.4. *Activity in vivo*

We have tested for B-domain transcriptional activity using a plasmid-borne HIS3 test gene (pGM3181) served by a promoter in which natural UAS elements at nt −70 had been truncated and replaced with the enhancer elements [3]. We tested histidine prototrophy and measured HIS3 RNA levels by Northern analysis. A single copy of the B-domain was quite active by both criteria in either plasmid-borne or chromosomally integrated constructs (section 2.1) (data not shown).

The 78 bp DE-S fragment (40 bp enhancer + 38 bp linker) was as active as the entire B-enhancer in integrated constructs (data not shown), and transcriptional activation of HIS3 test gene was strongly curtailed in a RAP1^{ts} mutant after 2 h at the non-permissive temperature (Fig. 5).

These findings demonstrate that RAP1 binds to a

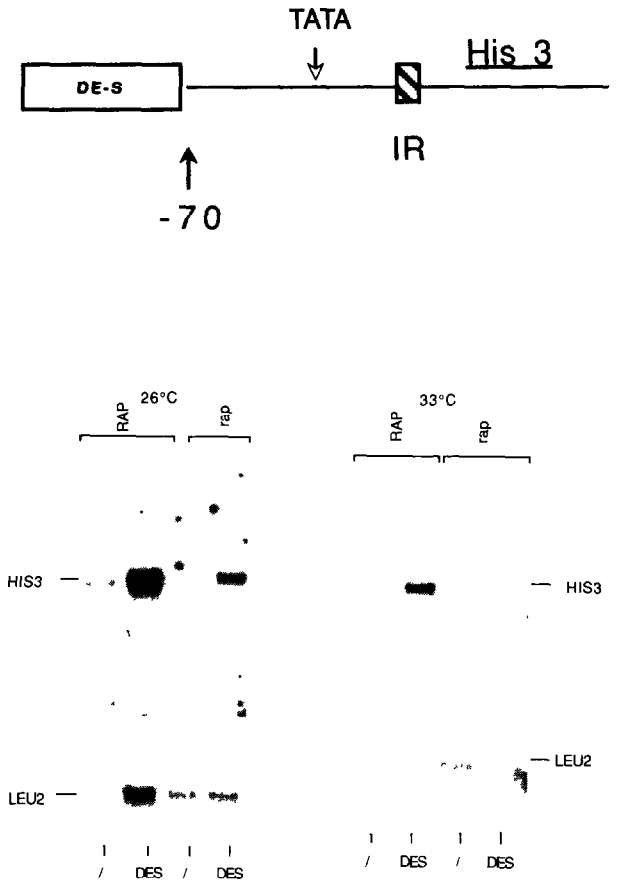

Fig. 5. Northern blot analysis showing dependence on RAP1 of transcriptional activation by the DES fragment in plasmid pGM3181 after chromosomal integration at the TRYP1 locus. (Top panel) Scheme of test gene used (for details see section 2.1 and [3]). (Bottom panel) Total RNA was prepared from yeast mutant rap1^{ts} and the RAP wt isogenic strain, run on 1% agarose, 6% formaldehyde gels and transferred to nitrocellulose. Probe for HIS3 was a uniformly ^{32}P-labelled single-stranded RNA probe. Quantity of RNA was normalized by hybridization to a labelled DNA probe homologous to the chromosomal LEU2 gene. Lanes '/' indicate transcription of the HIS3 test gene in the absence of enhancer sequences. Lanes 'DES' indicate transcription of HIS3 test gene driven by the DE-S fragment.

sequence in the Polyoma enhancer in vivo and is responsible for transcriptional activation in yeast cells.

4. DISCUSSION

There are now several examples of transcriptional regulatory elements from higher eukaryotes, including the Polyoma enhancer, that stimulate expression in transcriptional fusions in yeast, when substituted for native UAS elements [3,4,12]. Yeast proteins have been found to bind, in such chimaeric promoters, to the same motifs that are responsible for transcriptional activation in higher eukaryotes [12,19,20].

We show here that, under certain conditions, transcriptional activity of the Polyoma enhancer B-domain in yeast depends uniquely on the yeast multifunctional protein RAP1. This result was somewhat unexpected because, in yeast promoters, RAP1 is usually associated with other transcriptional activators such as GCN4 and ABF1 [15,21]. Furthermore, in a different promoter, we found that the B element is inactive, in single copy, in yeast, although it did significantly reinforce transcription when in multiple copies or in conjunction with the A-domain enhancer [3]. The effect of promoter context on the relative importance of the two domains of the Polyoma enhancer had been previously observed in mouse cells [2,22].

There has been little work on protein binding to the B element perhaps because, in most contexts, the entire B-domain is dispensable and has been considered of secondary functional importance. Careful studies of this region mostly concern the effect of mutations that extend the host-range of Polyoma to undifferentiated cells [23,24]. Also, this same domain is a strong transcriptional activator in mouse fibroblasts when artificially linked to the SV40 promoter and this activation is abolished upon removal of the 40 bp DE-S sequence (the *StuI–PvuII* fragment) [22].

So far, we have failed to detect mammalian proteins that footprint on the DE-S fragment RAP1 sequence, perhaps because HeLa nuclear extracts, which we have used as a source of mammalian DNA binding proteins, contain a predominant protein species whose footprint partially overlaps the RAP1 sequence (Pollice and Gilson, unpublished results) (Fig. 1B). Others have observed an identical footprint (Kryzke and Yaniv, personal communication).

EBP20, a rat liver nuclear protein that binds with high avidity to a primary site in the enhancer core region, also binds with lower avidity to the DES fragment and presents a DNaseI footprint that encompasses the RAP1 site [25]. It is also intriguing, that the Polyoma RAP1 site contains a pentanucleotide (CCAAT) which matches the consensus for binding of proteins of the NF1/CTF family that are implicated in replication as well as transcription. Interestingly, the 30 amino acid N-terminus of a member of this family presents a 30% identity with the C-terminus of RAP1 [26].

Although no mammalian homologue of RAP1 has been described in mammals or for that matter in any other organisms, the presence of RAP1-binding motifs

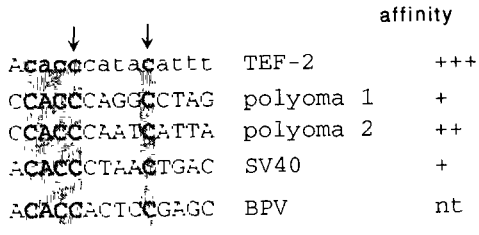

Fig. 6. Potential RAP1 binding sites in other viral enhancers. Symbols '+' indicate relative binding affinity, 'nt', not tested. The SV40 sequence is in the 'late' orientation.TEF-2 is from the UAS of the TEF2 gene, containing a high affinity RAP1 site.

in the SV40 and BPV enhancers (Fig. 6), suggests that there may indeed be such a protein. In fact we observed that purified RAP1 binds to the SV40 enhancer (data not shown).

The discovery of such a homologue would be potentially of great interest because of the central role this protein plays in yeast cells both in gene expression and in chromosome dynamics. RAP1 is a scaffold-bound [27] protein necessary for transcriptional activation of many house-keeping genes in yeast [28–30] as well as for silencing of the mating type genes and for telomeres stability.

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