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Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homolog and two contiguous proteins

(transcription factors/chromatin structure/DNA replication)

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ABSTRACT **The polyomavirus enhancer is composed of multiple DNA sequence elements serving as binding sites for proteins present in mouse nuclear extracts that activate transcription and DNA replication. We have identified three such proteins and their binding sites and correlate them with enhancer function. Mutation of nucleotide (nt) 5140 in the enhancer alters the binding site (TGACTAA, nt 5139–5145) for polyomavirus enhancer A binding protein 1 (PEA1), a murine homolog of the human transcription factor activator protein 1 (AP1). This mutation simultaneously reduces polyomavirus transcription and DNA replication. Reversion of this mutation simultaneously restores binding of PEA1 and both DNA replication and transcription. Binding of a second protein, PEA2, adjacent to the PEA1 site at nt 5147–5155 is enhanced by PEA1 binding, suggesting that these proteins interact. A third protein, PEA3, binds to the sequence AGGAAG (nt 5133–5138) adjacent to the PEA1 binding site; integrity of this late-proximal PEA3 binding site or an additional early-proximal site (nt 5228–5233) is important for enhancer function. We correlate binding of PEA1 and PEA2 with the induction of a DNase I-hypersensitive site in polyomavirus minichromosomes isolated from mouse fibroblasts.**

The polyomavirus enhancer activates the viral early promoter *in vivo* and is required for viral DNA replication (1–10). It is composed of multiple functionally redundant DNA elements whose activities vary with cell type and growth state (2, 5–7, 9, 11, 12). These elements serve as binding sites for cellular proteins (13–20) that most likely help form initiation complexes at cis-linked origins and promoters (5, 14, 21).

Two cellular proteins (15) bind to the polyomavirus enhancer at nucleotides (nt) 5139–5155 of the A3 strain (22) or nt 5114–5130 of the A2 strain (23). Polyomavirus enhancer A binding protein 1 (PEA1) binds to nt 5139–5145 (A2 strain nt 5114–5120), which make up the consensus sequence for the HeLa cell transcription factor activator protein 1 (AP1) (TGACTAA). AP1 activates the human metallothionein and simian virus 40 enhancers (24); it is most likely encoded by the human protooncogene c-*jun* (25), and it shares substantial sequence homology with the DNA-binding domains of yeast *GCN4* and avian v-*jun* (24–29). PEA1 is probably the murine homolog of AP1 (21, 30).

PEA2 binds to nt 5147–5154 (A2 strain nt 5122–5129), adjacent to the PEA1 binding site. An additional factor, polyomavirus enhancer B binding protein 1 (PEB1), binds to other enhancer sequences between nt 5180 and 5220 (A2 strain nt 5155–5195) (13, 14, 21), and several proteins—enhancer binding factor to polyomavirus element C (EF-C) and enhancer binding protein 20 (EBP20) (18–20) and polyomavirus enhancer D binding protein 1 (PED1) (J.P. and M.Y., unpublished observations)—bind to auxiliary enhancer elements.

Because of the functional redundancy of the polyomavirus enhancer, we chose to inactivate multiple important elements by introducing numerous random point mutations. Using this approach, we identified several polyomaviruses whose DNA replication and transcription were greatly reduced because of point mutations in the enhancer (5). In this report we provide evidence for the involvement of PEA1 and PEA2, with a third factor, PEA3, in the activation of both DNA replication and transcription by the polyomavirus enhancer. Furthermore, we correlate the binding of PEA1 and PEA2 with the formation of a DNase I-hypersensitive site in viral minichromosomes.

MATERIALS AND METHODS

Materials. Biotrans nylon membranes were purchased from ICN. NENSORB-20 cartridges were purchased from DuPont/New England Nuclear. All other materials were purchased from the best available sources.

Isolation of Nuclei and DNase I Digestions of Viral Chromatin. Nuclei were prepared from infected mouse cells at 24 hr or 40 hr after infection by lysis with 0.1% Nonidet P-40 in solution I (5 mM Pipes, pH 7.0/85 mM KCl/5% sucrose). Aliquots of nuclei were digested with DNase I at 7.5 μg/ml for 1 min at 20°C in solution I + 5 mM $MgCl_2$ and 5 mM $CaCl_2$, and the viral DNA was isolated by extraction of the digested nuclei (31, 32). To map DNase I-hypersensitive sites in viral minichromosomes, we used the indirect end-labeling technique (33). DNase I-digested viral DNA was digested with *Bam*HI and fractionated by electrophoresis on 1.5% agarose gels. The DNA was transferred (34) to nylon membranes and probed with the *Bam*HI–*Bcl* I fragment (nt 4657–5046) prepared by primer extension of a single-stranded M13–polyomavirus template. *Bam*HI-linearized viral DNA digested partially with *Hae* III provided markers to locate the positions of DNase I-hypersensitive sites as previously described (32).

***In Vitro* DNase I "Footprinting" Experiments.** Wild-type and mutant DNA radiolabeled fragments were digested with DNase I in the presence or absence of 3T6 cell nuclear extracts as previously described (14). *In vitro* DNase I-hypersensitive sites are numbered as defined in the text.

Gel Retardation Assays. Double-stranded oligonucleotide probes were labeled with [α-^{32}P]dATP by incubation with the Klenow fragment of *Escherichia coli* DNA polymerase and purified over NENSORB-20 cartridges. Nuclear extract from mouse 3T6 cells was incubated for 10 min at 20°C with 0.5 μg

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Abbreviations: nt, nucleotide(s); PEA1, -2, and -3, polyomavirus enhancer A binding proteins 1, 2, and 3; AP1, activator protein 1; PEB1, polyomavirus enhancer B binding protein 1; EF-C, enhancer binding factor to polyomavirus element C; HS, DNase I-hypersensitive site.

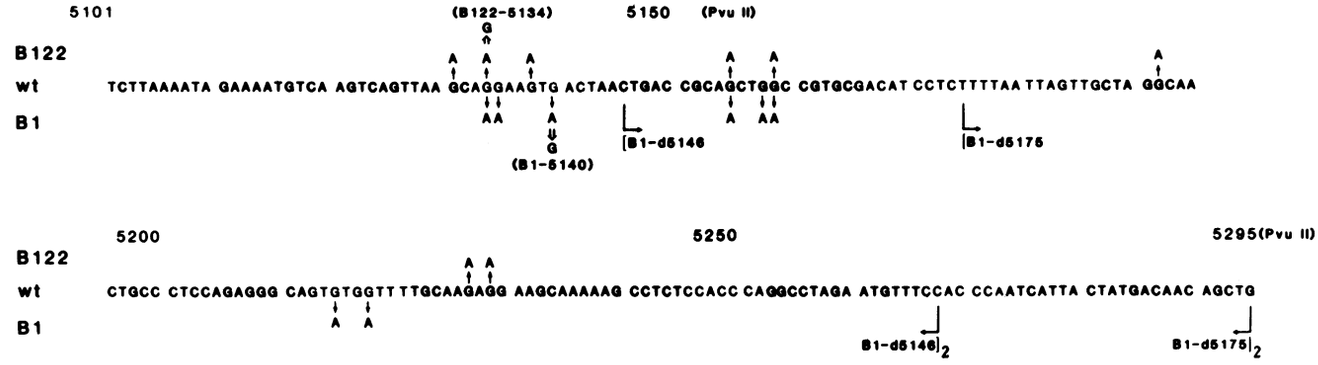

FIG. 1. Nucleotide sequence of polyomavirus enhancer [virus strain A3 numbering (22)]. wt, Wild type. Point mutations in mutant enhancers are indicated by single arrows. Sites of reversion are indicated by double arrows, or, in duplication revertants, by sequences enclosed by arrows.

of poly(dI-dC)·poly(dI-dC) and the appropriate competitor DNA (0.5 μg) in 0.02 ml of buffer C [10 mM Hepes, pH 8.0/17.5% (vol/vol) glycerol/0.1 mM EDTA/0.1 M NaCl/10 mM $MgCl_2$/2 mM dithiothreitol containing bovine serum albumin at 2 mg/ml. Radiolabeled probes (0.002 μg) were added and the samples were incubated for 30 min at 30°C. The samples were loaded on a 6% polyacrylamide gel and electrophoresed as previously described (15).

RESULTS AND DISCUSSION

Properties of Polyomavirus Enhancer Mutants and Revertants. The enhancers of polyomavirus mutants B1 and B122 are impaired in their capacity to activate viral DNA replication and early transcription (ref. 5; W.-J.T. and W.R.F., unpublished data) (Fig. 1, Table 1). Reversion at nt 5140 in mutant B1 or at nt 5134 in mutant B122 restores nearly wild-type levels of DNA replication and transcription to these viruses (B1-5140 and B122-5134; Fig. 1; Table 1). These sites of reversion occur within or near the binding site (TGACTAA) for the cellular protein PEA1 (13), suggesting that PEA1 is important for enhancer function. Duplication of sequences between nt 5146 and 5295 (Fig. 1) substantially restores replication to mutant B1 (5). These duplicated sequences include binding sites for PEA2, PEB1, and EF-C, suggesting that one of these or another protein can act synergistically to compensate for inactivating mutations near the PEA1 site.

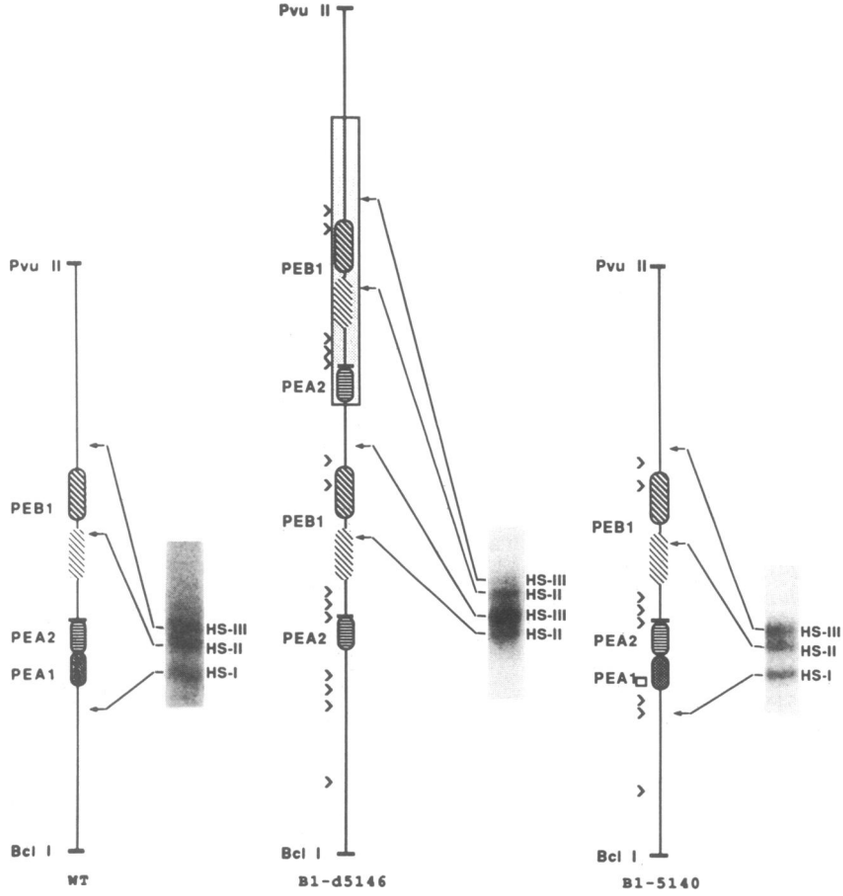

FIG. 2. DNase I hypersensitivity pattern of wild-type (WT) and mutant polyomavirus minichromosomes in mouse fibroblasts. DNase I-hypersensitive sites are labeled HS-I (≈ nt 5125), HS-II (≈ nt 5185), and HS-III (≈ nt 5235). Polyomavirus strain A3 factor binding sites (depicted by ovals) are as follows: PEA1, nt 5139–5145; PEA2, nt 5146–5154; PEB1, nt 5170–5218 (14, 15). Mutations in B1-d5146 and B1-5140 are indicated by carats (v). Duplicated region in B1-d5146 is boxed. Site of reversion (nt 5140) in B1-5140 is shown by a small open box.

Table 1. Ability of viral enhancers to activate transcription and DNA replication

Virus	Transcription, %	Replication, %
Wild type	100	100
B1	0–10	0–10
B1-5140	50–80	50–80
B122	10–50	0–10
B122-5134	50–80	50–80

Activation of transcription or DNA replication was measured in transient assays by transfection of DAP-3 or 3T6 cells (5).

Effect of Enhancer Mutations on Factor Binding *in Vivo*. To detect factors bound to the polyomavirus enhancer *in vivo*, we examined the DNase I-hypersensitive sites (HSs) in viral minichromosomes (Fig. 2). In wild-type minichromosomes, DNase I hypersensitivity at HS-I is induced near binding sites for PEA1 and PEA2 (15, 34, 35). HS-II may be induced by PEB1 binding, since *in vitro* footprints of PEB1 are DNase I hypersensitive in this region (ref. 14; see also Fig. 4). HS-III may be induced by an as yet undetected factor that binds to a site between nt 5220 and 5250.

We were unable to obtain reliable preparations of B1 virus minichromosomes to determine how HS-I was altered by its mutations near the PEA1 binding site. Revertant B1-d5146, however, retains all of the B1 virus mutations within its unique and duplicated sequences and grows well, so we determined the DNase I hypersensitivity pattern of its minichromosomes. The clear absence of HS-I from B1-d5146 minichromosomes (Fig. 2) and from a similar duplication revertant, B1-d5175 (data not shown), indicates that HS-I would not be present in B1 minichromosomes. HS-II and HS-III are present and duplicated in these revertants as expected (Fig. 2). Reversion at nt 5140 restores HS-I in revertant B1-5140 minichromosomes (Fig. 2), indicating that restitution of the PEA1 binding site restores both enhancer function and HS-I *in vivo*.

Effect of Enhancer Mutations on Factor Binding *in Vitro*. DNase I footprints of wild-type DNA incubated with nuclear extract demonstrate protection of the PEA1 and PEA2 binding sites and induction of DNase I hypersensitivity at nt 5133 and 5136 (Fig. 3, lanes a–c; ref. 15). DNase I footprints of mutant B1 DNA incubated with nuclear extract indicate that PEA1 fails to bind this DNA and PEA2 binding is considerably weakened (Fig. 3, lanes d–f). Similar results were obtained with the duplication revertant B1-d5146 DNA, whose sequence in this region is identical to that of B1 (not shown). Reversion of nt 5140 within the PEA1 binding site restores binding of PEA1 and enhances PEA2 binding to B1-5140 DNA *in vitro* (Fig. 3; lanes g–i). The mutations at nt 5215 and 5218 in mutant B1 have no apparent effect on binding of PEB1 *in vitro* (Fig. 3; lanes d–f) (unpublished observations). These observations correlate the binding of PEA1 and PEA2 *in vitro* with enhancer function and the formation of HS-I in viral minichromosomes.

Evidence for a Third Protein (PEA3) Binding Adjacent to the PEA1 Site. The mutations in virus B122 greatly reduce DNA replication (Table 1) but have no effect on the formation of HS-I in viral minichromosomes (data not shown) or on binding of PEA1, PEA2, or PEB1 *in vitro* (Fig. 4, lanes a and

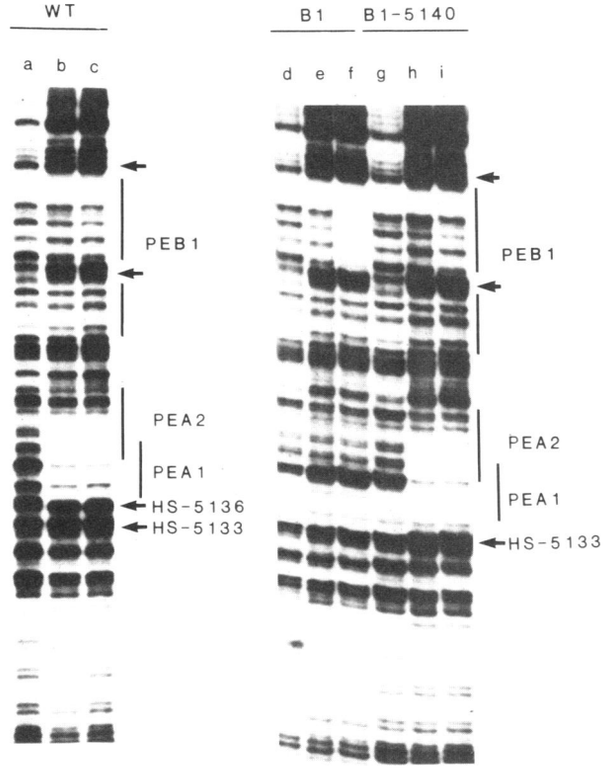

FIG. 3. *In vitro* DNase I footprints of wild-type (WT), B1, and B1-5140 viral DNAs. Products of DNase I digestion in the absence of 3T6 cell nuclear extract are shown in lanes a, d, and g. Products of DNase I digestion in the presence of 1× and 2× amounts of 3T6 cell nuclear extract are shown in adjacent lanes (WT DNA, lanes b and c; B1 DNA, lanes e and f; B1-5140 DNA, lanes h and i). Protected sequences are indicated by solid vertical bars. DNase I-hypersensitive sites are indicated by arrows. The site of reversion in B1-5140 is within the region protected by PEA1.

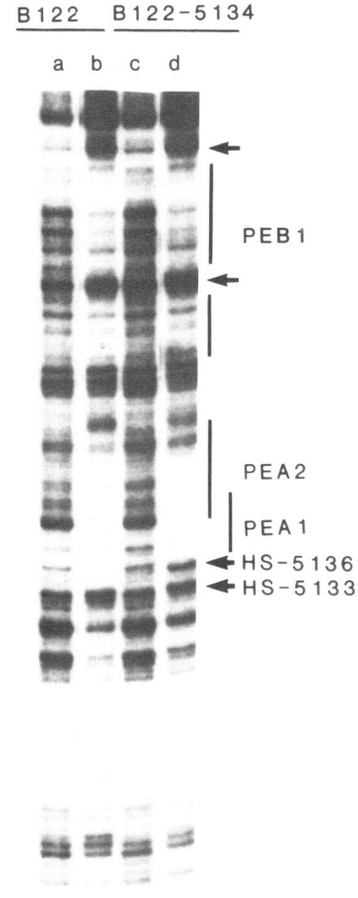

FIG. 4. *In vitro* DNase I footprints of mutants B122 and B122-5134 DNAs. Products of DNase I digestion in the absence of 3T6 cell nuclear extract are in lanes a and c; products of DNase I digestion in the presence of nuclear extract are in lanes b and d. Protected sequences are indicated by vertical bars. Hypersensitive sites are indicated by arrows.

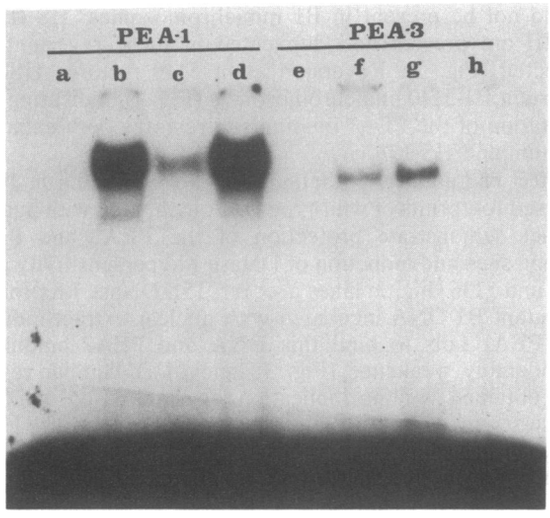

FIG. 5. Gel retardation assays. DNA–protein complexes with radiolabeled PEA1 oligonucleotide are in lanes a–d; DNA–protein complexes with radiolabeled PEA3 oligonucleotide are in lanes e–h. No nuclear extract was present in lanes a and e; 10 μg of 3T6 cell nuclear extract was present in lanes b–d and f–h. Unlabeled competitor oligonucleotides were PEA1 in lanes c and g and PEA3 in lanes d and h. "Upper" strands of oligonucleotides used were

5′-TCGAGTGACTAACTCGA-3′
PEA1
5′-TCGAGCAGGAAGTTCGA-3′.
PEA3

b). Footprints of wild-type viral DNA exhibit DNase I hypersensitivity at nt 5133 and 5136 (Fig. 3, lanes a–c). The footprint of B122 DNA reveals that HS-5136 is absent and that reversion of nt 5134 in B122-5134 DNA restores this site (Fig. 4). DNase I footprints of mutant B1 DNA do not display either hypersensitive site, and reversion of nt 5140 restores only HS-5133 (Fig. 3, lanes d–i). Footprints of wild-type DNA in the presence of a competitor oligonucleotide containing the sequence AGGAAG lack HS-5136, suggesting that a soluble factor binds to this sequence (data not shown). This suggests that HS-5136 is not induced by PEA1 or PEA2, and it may result from a third protein (PEA3) binding within this region.

To determine whether a PEA3 protein binding to nt 5133–5138 can be detected in 3T6 cell nuclear extracts, we performed gel retardation assays (36), using a radiolabeled double-stranded oligonucleotide probe containing this putative PEA3 binding site (AGGAAG) (Fig. 5). A protein forms a complex with this probe (lane f); complex formation is blocked by competition with excess unlabeled PEA3 oligonucleotide (lane h) but not by excess unlabeled PEA1 oligonucleotide (lane g). With the same extract, when a radiolabeled PEA1 probe (TGACTAA) (Fig. 5) is used, the PEA1 protein forms a complex with the PEA1 probe (lane b); complex formation is blocked by competition with excess unlabeled PEA1 oligonucleotide (lane c) but not by excess unlabeled PEA3 oligonucleotide (lane d). These experiments clearly indicate that in 3T6 cell nuclear extract there is a PEA3 protein whose binding site includes the sequence AGGAAG. The large difference in band intensity between the PEA1 complex and the PEA3 complex suggests that PEA3 is present at lower levels, or that PEA3 binds to DNA less tightly than PEA1, and may explain the lack of protection of AGGAAG sequences in DNase I footprints. Hypersensitivity is more readily detected in footprints than is protection against DNase I, when factor binding is weak or when the concentration of factor is limiting and occupancy is low (37).

The sequence element containing the PEA3 binding site is duplicated in various laboratory isolates of polyomavirus and is present in the enhancers of several adenovirus *E1A* genes and the major late transcription unit, in several retroviral long terminal repeats, in the BK virus enhancer, and in the human β-interferon enhancer (5, 38–41). Furthermore, we have detected binding to the PEA3 site in human 293 cell nuclear extracts (M.E.M. and W.R.F., unpublished observations). In addition, HeLa cell nuclear extracts contain a factor that binds to this sequence element in the adenovirus 5 *E1A* enhancer and

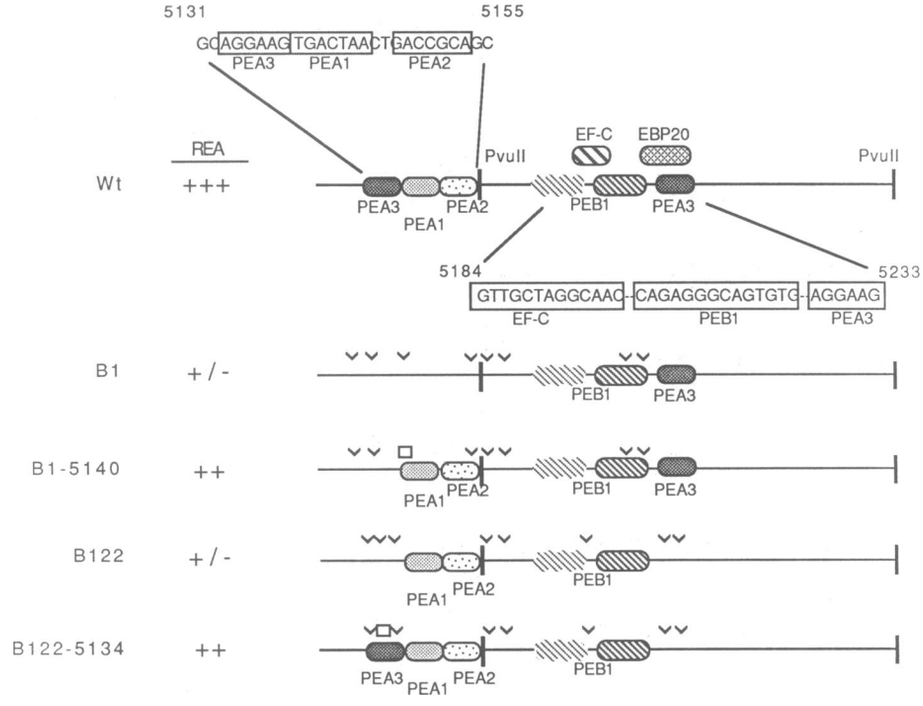

FIG. 6. Cellular factors that bind to polyomavirus enhancers. Positions of cellular factor binding sites within the polyomavirus enhancer are depicted by ovals. Wild-type (wt) sequences important for factor binding (as described in text) are displayed within boxes. Relative enhancer activity (REA) was derived from Table 1. Positions of mutations are indicated by carats (v). Sites of reversion are shown by small open boxes.

homologous polyomavirus sequences compete for this binding (P. Hearing and J. Bruder, personal communication).

A second sequence to which the factor PEA3 might bind occurs in the polyomavirus enhancer near the PEB1 site between nt 5228 and 5233 (Fig. 1). Virus B122 mutations at nt 5134 (late-proximal PEA3 site) and 5229 (early-proximal PEA3 site) alter the same residue within both putative PEA3 sites. This second PEA3 binding site in the polyomavirus enhancer may help explain the functional differences between viruses B1, B1-5140, B122, and B122-5134 (Fig. 6). B1 is not bound by PEA1, PEA2, or PEA3 at the late-proximal enhancer element and, as a result, lacks any measurable enhancer function. The revertant of mutant B1, B1-5140, is bound only by PEA1 and PEA2 near the late-proximal PEA3 site; however, the early-proximal PEA3 site remains intact in this virus and its enhancer functions at nearly wild-type levels. The functional defect of the mutant B122 enhancer, which has both PEA3 sites altered, indicates that binding of PEA1 and PEA2 is not sufficient for enhancer function. Reversion of nt 5134 restores the late-proximal PEA3 site in virus B122-5134, resulting in nearly wild-type enhancer activity, and indicates that at least one intact PEA3 site is required for activation of the enhancer by PEA1 and PEA2.

Activation of Viral and Cellular Enhancers. Since PEA2 binding to its site is influenced by PEA1 binding (Fig. 3), we suggest that a complex of PEA1, PEA2, and possibly PEA3 activates the polyomavirus enhancer in mouse fibroblasts. Binding of PEB1 and of PEA3 and possibly of other factors to sequences between nt 5210 and 5233 appears to compensate for mutations that affect this complex in the duplication revertants of mutant B1.

PEA1, PEA2, and PEA3 undoubtedly function in the activation of cellular enhancers during development and differentiation. Neither PEA1 nor PEA2 is detected in extracts from undifferentiated F9 cells (21). Upon differentiation, PEA1 binding is induced and F9 cells become permissive to viral infection (1, 21). In quiescent NIH 3T3 cells PEA1 binding is induced by treatment with phorbol esters and by simian virus 40 transformation (30), suggesting a role for PEA1 in cellular proliferation and oncogenesis.

Since polyomavirus middle tumor (T) antigen and large T antigen alter the activities of several cellular kinases (42–45), it is likely that they activate PEA1 and other enhancer binding factors during viral growth and in transformed cells. This might explain how the polyomavirus enhancer is activated by the Ha-*ras* gene and by phorbol 12-tetradecanoate 13-acetate (45, 46), and why middle T antigen is capable of transactivating numerous promoters controlled by enhancers (47). A report published after review of this manuscript (48) indicates that the PEA1 binding site is a target of Ha-*ras*, phorbol 12-tetradecanoate 13-acetate, and serum-stimulated transcription.

Our observation that PEA1 is involved directly in the activation of polyomavirus DNA replication suggests that oncogenes in this family need not act through transcription to transform cells. They may act directly upon the replicational apparatus of the cell.

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