A Polyomavirus Enhancer-Binding Protein, PEBP5, Responsive to 12-O-Tetradecanoylphorbol-13-Acetate but Distinct from AP-1

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Element I, homologous to the adenovirus type 5 E1A enhancer core, is a 10-bp sequence in the A core of the polyomavirus enhancer and was shown previously to be responsive to 12-O-tetradecanoylphorbol-13-acetate (TPA). We found that element I by itself was capable of activating polyomavirus DNA replication in COP-5 cells which express the polyomavirus large T antigen. A nuclear factor, polyomavirus enhancer-binding protein 5 (PEBP5), which bound to the entire sequence of element I and was responsive to TPA was identified by an in vitro binding assay. Although the binding site of PEBP5 partly overlaps with that of PEBP1 (PEA1), a member of the AP-1 family, PEBP5 appears to be a distinct factor. Since we previously showed that element I alone was able to activate transcription, our present results suggest that PEBP5 is involved in the regulation of both transcription and replication of DNA. The amount of PEBP5 increased after F9 cells were induced to differentiate by retinoic acid. A relatively large amount of PEBP5 was detected in lymphoid and trophoblast cells.

The polyomavirus (Py) enhancer is a useful tool for the study of the regulation of gene expression. Although an enhancer was originally identified as a cis-acting element to stimulate transcription, it has also been shown to be required for Py DNA replication (5). The observation that the Py enhancer is required for viral DNA replication at the two-cell stage of the mouse embryo but is not in a one-cell embryo makes this system valuable for exploring some aspects of developmental regulation (21).

The Py enhancer is contained in a 244-bp segment of the Py genome, from the BclI site (nucleotide [nt] 5021) to the origin proximal to the PvuII site (nt 5128). By deletion analysis, Veldman et al. have identified four distinct regions within this segment: the A, B, C, and D elements (42). Of these, the A and B elements can function by themselves and hence constitute the core of the enhancer (42). Studies by Mueller and others have revealed three functional elements, 1, 2, and 3, for transcriptional activation (25) and the α and β cores for replication activation (26, 27). Elements 2 and 3 or the α and β cores roughly correspond to Veldman’s A and B elements, respectively. We have been studying the function of the A element intensively, since it has several interesting properties. First, the A element alone is sufficient to activate both transcription and DNA replication (42). In addition, it is inactive in F9 cells but becomes functional after F9 cells are induced to differentiate (13, 17) and is the target of transcriptional activation by several oncogene products and a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) (15, 32, 37, 45, 46, 48).

The A core element is a 24-bp region containing three sequence motifs (Fig. 1, WA). Two nuclear factors, polyomavirus enhancer-binding protein 1 (PEBP1) and PEBP2 (or PEA1 and PEA2, respectively [33]), which recognize two adjacent motifs in the A core element (37, 48) have been identified. The binding site of PEBP1 is the consensus sequence for the binding of the activator protein, AP-1 (1, 18). The third motif is a 10-bp sequence partly overlapping the PEBP1 binding site and is homologous to the enhancer core of adenovirus type 5 E1A, termed element I (12). Veldman’s A element will subsequently be called the A core, since we will deal with element I within the A core.

The importance of element I in Py enhancer function is indicated by several lines of evidence. For example, the enhancers of Py mutants capable of growing in PCC4 cells manifest duplication of a segment which corresponds almost exactly to element I (23). Tang et al. (40) have shown that a Py mutant having multiple base changes in the enhancer region is growth defective, but a single base change within element I to the wild-type residue restores the growth capability of the virus. What makes element I particularly interesting to study is that it is a target of transcriptional activation by TPA (48) or by the activated Ha-ras gene (37), as is the case for the PEBP1 (PEA1) binding site. More recently, several other nonnuclear oncogenes were shown to activate the function of element I as well (43). A factor named PEA3 interacting with element I has been reported (20). However, neither the precise recognition sequence nor the relationship to other A core-binding factors has been clearly documented for the element I-binding factor.

In the present study, we found that element I was able to activate Py DNA replication and we identified a TPA-responsive factor, termed PEBP5, most likely to be responsible for that function.

MATERIALS AND METHODS

Plasmids. The plasmid, PyΔ3·CAT, was a gift from R. Kamen (42), and the XhoI site therein was converted to a BglII site by linker insertion. Into this BglII site of pyΔ3·CAT, we inserted various DNA fragments or synthetic oligonucleotides (Fig. 1), which represent portions of the polyomavirus enhancer sequence. The designations of plasmids and inserted fragments were as follows: pPy·CAT, the entire 244-bp enhancer fragment BPP from the BclI (nt 5021) to the PvuII (nt 5265) site; pWA·CAT, the WA oligonucleotide, which represents the wild-type A core sequence; p(WA)2·CAT, a head-to-tail dimer of the WA

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FIG. 1. Structure of plasmids used for the replication assay. pPyΔ : CAT contains polyomavirus DNA from the BamHI site (nt 4632) to the BglII site (nt 152) except for the enhancer region from the BclI site (nt 5021) to the PvuII site (nt 5265). This segment of polyomavirus DNA harbors the regulatory elements including the early promoter, the replication origin, and large-T-antigen-binding sites. The plasmid also contains the fragment of pSV2CAT (10) spanning the chloramphenical acetyltransferase (CAT) gene (open box), the intron and polyadenylation signal of simian virus 40 (double line), and the fragment of pBR322 carrying the ampicillin resistance gene (solid line). At the position from which the enhancer region was removed, a BglII site was created. Shown below are the polyomavirus enhancer fragment and its subfragments, each of which were inserted into the BglII site of pPyΔ : CAT. Py represents the whole enhancer region from the BclI site to the origin-proximal PvuII site, where the A and B cores are indicated. WA and WEI are oligonucleotides representing the A core or element I sequence, respectively. The binding sites for PEBP1 and PEBP2 are indicated by the solid lines below the sequence (37, 48). M3A (48) and MEI are mutated versions of WA and WEI oligonucleotides, respectively, with 2 bp changes within element I as indicated. WA, WEI, M3A, and MEI have the BamHI sticky end at the late side and the BglII sticky end at the early side of the molecule. The trimers of WEI and MEI oligonucleotides were chemically synthesized with a 3-bp spacer, GAT, between the elements.

oligonucleotide; pM3A : CAT, the M3A oligonucleotide, the A core sequence containing two base substitutions in element I (48); p(M3A)2 : CAT, a head-to-tail dimer of the M3A oligonucleotide; pWEI : CAT, the WEI oligonucleotide, which represents the wild-type element I sequence; pMEI : CAT, the element I sequence containing the same two base substitutions as in the M3A oligonucleotide; p(WEI)3 : CAT, a head-to-tail trimer of the WEI oligonucleotide; and p(MEI)3 : CAT, a head-to-tail trimer of the MEI oligonucleotide. Each oligonucleotide was constructed with a BamHI sticky end at the late side and a BglII sticky end at the early side. Dimer constructs, therefore, had a 5-bp spacer between the units when multimerized. The trimer constructs of WEI and MEI sequences were chemically synthesized with a 3-bp spacer, GAT, between the units.

The number and the orientation of inserted sequences were determined by restriction enzyme mapping and dideoxy sequencing methods (35) by using denatured plasmid templates (11). As an internal control plasmid, pUC13 was prepared from the DNA adenine methylation-defective dam-minus strain of Escherichia coli (dam-3 [19]).

Cells. Mouse F9, C127, COP-5 (a derivative of C127 which is transformed by replication-defective polyomavirus DNA [41]), trophoblast, and NIH 3T3 cells were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum (calf serum in the case of NIH 3T3 cells), 100 U of
penicillin per ml, and 100 μg of streptomycin per ml. To induce differentiation, F9 cells were treated with 1 μM of retinoic acid for 5 days (39). Mouse P388D1 (macrophage cell line), L1210 (B-cell line), and EL4 (T-cell line) cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Replication assay. On the day preceding transfection, 1.5 × 10⁶ cells were plated on 100-mm (diameter) dishes. Sub-confluent cells were transfected with plasmid DNAs by the DEAE-dextran method as described previously (42). Briefly, each culture dish was cotransfected with various amounts of DNA ranging from 0.25 to 12 μg of test plasmid DNA or with 10 ng of control pUC13 DNA in 2.5 ml of Tris-saline buffer and once with phosphate-buffered saline and fed with fresh medium. Twenty-four hours after transfection, low-molecular-weight DNA was isolated by the Hirt extraction method (14) and extracted with phenol, and B-PEA3 oligonucleotide fragment from the EcoRI site to the polyomavirus enhancer of the dried membrane to X-ray film at −70°C. The digested DNA was separated by DEAE-dextran method as described previously (42). Briefly, 10 ng of control pUC13 DNA in 2.5 ml of Tris-saline buffer containing 300 μg of DEAE-dextran per ml. After 30 min of incubation at 37°C in 10% CO₂, dishes were washed once with Tris-saline buffer and once with phosphate-buffered saline and fed with fresh medium. Twenty-four hours after transfection, low-molecular-weight DNA was isolated by the Hirt extraction method (14) and extracted with phenol, and B-PEA3 oligonucleotide fragment from the EcoRI site to the polyomavirus enhancer of the dried membrane to X-ray film at −70°C. The digested DNA was separated by electrophoresis through a 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized with nick-translated [α-32P]P-labeled pPyΔ ∙ CAT DNA, and autoradiograms were made after exposure of the dried membrane to X-ray film at −70°C.

Mobility shift assay. Oligonucleotides representing the polyomavirus enhancer A core, element I, their mutated versions (Fig. 1; see Fig. 5A), or the PEBP1 binding site

5'-GATCCAGTGGACTACTA
GTCACGATTGATCTAG-5'

and having the BamHI sticky end at the late side and the BglII sticky end at early side were inserted into the BamHI site of pUC13. The EcoRI-HindIII fragment was excised and used as a probe by labeling the 5' terminus with γ-32P]PATP and T4 kinase. Chemically synthesized oligonucleotides with BamHI-BglII sticky ends prior to insertion into pUC13 were used as unlabeled competitors unless specified otherwise. In the case of a single copy of the PEBP1 binding site, a fragment from the EcoRI site to the HindIII site of pUC13 containing a single copy of the PEBP1 site was used as a competitor. In the case of a trimer of the chemically synthesized PEBP1 binding site, a head-to-tail trimer of the PEBP1 oligonucleotide shown above was used. Each PEA3 oligonucleotide

5'-TCGAGCAGGAAGTCGA
AGTCTGCTCTTCAAGCTTCTCAG-5'

and B-PEA3 oligonucleotide

5'-TGCAGAGGACAGGAAAA
AGGTCTCCTCTGATTTG-5'

was used as a monomer, whereas the hexanucleotide

5'-AGGAAG
TCCTTC-5'

was used as a head-to-tail trimer in the competition experiment (see Fig. 8). Nuclear extracts from cells were prepared by the method of Dignam et al. (6). The binding reaction between the nuclear extract and the probe was performed in 15 to 24 μl of Tris-glycerol buffer or in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)·Ficoll buffer in the presence of 0.2 to 1.0 μg of poly(di-l-dC) per μg of nuclear extract protein with or without 0.5 μg of sheared salmon sperm DNA per μg of nuclear extract protein at room temperature for 30 min. Tris-glycerol buffer contained 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 1 mM EDTA, and HEPES·Ficoll buffer contained 20 mM HEPES (pH 7.6), 4% Ficoll, 1 mM MgCl₂, 40 mM KCl, 0.1 mM EGTA [ethylene glycol-bis(β-aminopropylether)-N,N,N',N'-tetraacetic acid], and 1 mM dithiothreitol. In the experiments shown in Fig. 3 and 10, Tris-glycerol buffer was used, whereas HEPES·Ficoll buffer was used for experiments shown in Fig. 5, 6, 8, 9, and 11. Both buffer systems gave essentially the same results in the mobility shift assay. The DNA-protein complexes were separated from the free DNA probe on 6 to 8% polyacrylamide gels (39:1, acrylamide/bisacrylamide ratio), and dried gels were exposed at −70°C to X-ray film.

Methylation interference footprinting. An asymmetrically end-labeled DNA fragment in which G and A residues were specifically modified by dimethyl sulfate treatment by the method of Maxam and Gilbert (22) was used as a probe in the mobility shift assay as described above. The complexes and free forms separated by electrophoresis were localized by exposing the wet gel to X-ray film at 4°C overnight. DNAs were eluted electrophoretically from the gels and purified by passing the eluates through a NENSORB (Dupont, NEN Research Products). Recovered DNAs were incubated in 20 mM ammonium acetate and 0.1 mM EDTA (pH 7.6) at 90°C for 15 min. After the addition of a one-ninth volume of 10 M piperidine, DNA samples were heated at 90°C for an additional 30 min. An equal amount of radioactivity from complexed or free-form DNA was loaded in each lane of an 8% polyacrylamide gel (39:1, acrylamide/bisacrylamide ratio)-urea sequencing gel. Gels were dried and exposed to X-ray film at −70°C.

RESULTS

Element I itself can activate Py DNA replication. Element I by itself was shown previously to activate transcription (48). Here, we examined the ability of element I to activate Py DNA replication. The plasmid pPyΔ ∙ CAT is a construct which harbors the Py replication origin core but lacks the enhancer region from the BclI site at nt 5021 to the PvuII site at nt 5265 (Fig. 1). When pPyΔ ∙ CAT was transfected into COP-5 cells, a very low level of plasmid replication was observed (Fig. 2A, lane 1, and 2B, lane 1). This level was considered to be the basal level of replication of the plasmid without an enhancer. In the experiment described in the legend to Fig. 2, test plasmids were always cotransfected with pUC13 plasmid DNA prepared from a dam methylase-defective strain of E. coli as an internal control to monitor the transfection and extraction efficiency. Insertion of the entire enhancer fragment into pPyΔ ∙ CAT restored the replication ability (Fig. 2A, lane 1), indicating the enhancer dependency of Py DNA replication. It has been shown that the A core of the Py enhancer has the ability to activate Py DNA replication (42). Oligonucleotide WA (Fig. 1) representing the A core was inserted into pPyΔ ∙ CAT as a monomer or a dimer. As seen in Fig. 2A, lanes 3 and 4, both the monomer and the dimer of the A core activated Py DNA replication, and the activity of the latter was some 5 to 20 times as high as that of the former, depending on the experiment (Fig. 2B, lanes 2 and 4). The activity achieved by
the dimer of WA was about 30% that of the whole enhancer (Fig. 2A, lanes 1 and 4). We then introduced the two base changes within element I in the A core (M3A oligonucleotide in Fig. 1) (48). These mutations greatly reduced the replication-enhancing activity of the A core (Fig. 2B, lanes 3 and 5). As reported previously, the base changes in M3A were located outside of the PEBP1 and PEBP2 binding sites (48). These results showed that element I is an important component for A core function in the activation of DNA replication. It must be noted that the mutations did not abolish A core function completely, suggesting the involvement of the remaining two motifs for A core function.

Next, we tested directly whether element I alone could activate pPyΔ·CAT replication. The monomer of element I slightly increased the replication activity (approximately 50%) above the basal level (Fig. 2B, lanes 1 and 6). This slight increase was abolished by the mutations in element I (lanes 6 and 7). The trimer of element I enhanced the replication significantly (four- to fivefold; lane 8), suggesting a synergistic effect of the element when multimerized. The mutations in element I abolished this activity (lane 9). As will be shown below, we confirmed that only the element I binding protein, PEBP5, but not PEBP1 bound element I even when it was trimerized.

From the above results, we concluded that element I constituted a functional unit and was one of the major functional units of the A core necessary for activation of Py DNA replication.

The replication of Py DNA that we observed was dependent on Py large T antigen, since the plasmid containing a dimer of the wild-type A core replicated in COP-5 cells but not in C127 cells, the parental cell line, which does not express Py T antigens (data not shown). We confirmed by immunoprecipitation that COP-5 cells expressed the Py large T antigen (data not shown).

**Nuclear factor interacting with element I.** The protein factor interacting with element I was searched for by the mobility shift assay. As will be shown below, the choice of carrier DNA in the binding reaction was found to be crucial to revealing adequate complex formation. In general, the use of sheared salmon sperm DNA as a nonspecific competitor eliminated the binding of PEBP1, while it had little effect on PEBP5 binding. Salmon sperm DNA also reduced the general background significantly (compare lanes 1 of Fig. 3B and C). In the following experiments, a mixture of poly(dI·dC) and salmon sperm DNA was used when PEBP5 was characterized, while poly(dI·dC) alone was used when both PEBP1 and PEBP5 were to be characterized. When the nuclear extract of COP-5 cells was incubated with the probe DNA containing the element I sequence, a single band was detected (Fig. 3, lane 1). This complex formation was abolished by the addition of the unlabeled monomer (data not shown) or trimer of element I oligonucleotide ([MEI]3) in a dose-dependent manner (lanes 2 to 4), but not by a monomer (data not shown) or trimer of the element I oligonucleotide of the mutated sequence ([MEI]3) (lanes 5 to 7). The factor binding specifically to element I was named PEBP5, according to the rule we described previously (48).

**PEBP5 binding sequence.** To determine the exact location of PEBP5 binding, we carried out methylation interference footprinting on the PEBP5 element I complex (Fig. 4). On the early RNA strand (Fig. 4, lanes 1 to 3), the methylation of the two G residues and one A residue spanning the region from nt 5109 to 5111 interfered with PEBP5 binding strongly, whereas that of the remaining three A residues and two 3' G residues showed weak interference. On the late RNA strand...
FIG. 3. Mobility shift assay with the COP-5 cell nuclear extract and element I probe. A total of 10^4 cpm of 32P-labeled WEI probe (specific activity, 5 × 10^7 cpm/μg) was incubated with 4.6 μg of COP-5 cell nuclear extract protein in the presence of poly(dI-dC) and salmon sperm DNA. The following oligonucleotides were added to the reaction mixture as competitors: none (lane 1), a trimer of WEI (lanes 2 to 4), and a trimer of MEI (lanes 5 to 7). The amount of competitor DNA relative to that of WEI probe is shown in fold molar excess above each lane. Complexed and free forms of the probe are indicated. Polyacrylamide (6%) gel was used.

(lanes 4 to 6), the methylation of the A residue at nt 5114 interfered with the binding of PEBP5 strongly, but that of the two G residues (nt 5107 and 5117) did so only weakly. In addition, on the late RNA strand, the methylated G residue just outside (3' to) element I (arrow) showed the augmentation of PEBP5 binding. Therefore, the recognition sequence of PEBP5 covered almost the entire element I and, at the same time, PEBP5 appeared to contact more closely the bases on the late side of element I (Fig. 4, bottom).

To further confirm the recognition sequence of PEBP5 determined by footprinting and also to examine the relationship of its binding site with those of other A core-binding factors, we used the A core sequence as a probe in the following mobility shift assays. Wild-type as well as the mutated A core sequences were employed in which M1A, M2A, or M3A harbored mutations in the PEBP1 binding site, PEBP2 binding site, or element I, respectively (Fig. 5A). Mutations in M4A were located in the region in which both element I and the PEBP1 binding site overlapped. When the wild-type A core was used as a probe and a mixture of salmon sperm DNA and poly(dI-dC) were used as a carrier to detect PEBP5, two complexes marked IB and II (designated to be consistent with the band names in Fig. 5C) were formed with the extract of COP-5 cells (Fig. 5B, lanes 1 and 16). Various unlabeled oligonucleotides were then included as competitors in the binding reaction. The oligonucleotides (WEI), WA, M1A, and M2A virtually abolished the formation of band IB (lanes 2 and 3, 6 and 7, 8 and 9, and 10 and...
FIG. 5. Mobility shift assay with wild-type and mutated A core probes. (A) Base pair changes introduced in the A core. WA is the wild-type A core. The positions of altered bases are as follows: in the PEBP1 binding site outside of element I (M1A), in the PEBP2 binding site (M2A), in element I outside of the PEBP1-binding site (M3A), and in the region in which element I and the PEBP1 binding site overlap (M4A). (B) Mobility shift assay with wild-type and mutated element I or A core as competitors. A total of 1.2 × 10^6 cpm of ^32P-labeled WA probe (specific activity, 1.2 × 10^6 cpm/μg) was incubated with 4.6 μg of COP-5 cell nuclear extract protein in the presence of poly(dI-dC) and salmon sperm DNA. Oligonucleotides were added to the reaction mixture as competitors, as shown above each lane. Abbreviations: (WEI), a trimer of the WE1 sequence; (MEI), a trimer of the ME1 sequence. The amount of competitor DNA relative to that of WA probe was (in fold molar excess) 50 (lanes 2, 4, 6, 8, 10, 12, and 14) or 500 (lanes 3, 5, 7, 9, 11, 13, and 15). See the text for a description of bands IB and II. Polyacrylamide (6%) gel was used. (C) Mobility shift assay with wild-type and mutated A core probes. A total of 1.7 × 10^6 cpm of each probe labeled with ^32P was incubated with 4.6 μg of COP-5 cell nuclear extract protein in the presence of poly(dI-dC). Probes are shown above each lane. Specific activities of all the probes were almost the same (ca. 6 × 10^6 cpm/μg). See the text for a description bands IA+II, IA, IB, and II. Polyacrylamide (6%) gel was used.
FIG. 6. Mobility shift assay with M3A (A) and the PEBP1 site (B) as probes and a trimer of WEI as a competitor. (A) A total of 10^6 cpm of ^32P-labeled M3A probe (specific activity, 10^8 cpm/µg) was incubated with 2.0 µg of COP-5 cell nuclear extract protein in the presence of poly(dI-dC). Oligonucleotides were added to the reaction mixture as competitors, as shown above the lanes. Abbreviations: (WEI), a trimer of WEI; (MEI), a trimer of MEI. The amount of competitor DNA relative to that of M3A probe was (in fold molar excess) 5 (lanes 2, 6, and 10), 25 (lanes 3, 7, and 11), 125 (lanes 4, 8, and 12), or 625 (lanes 5, 9, and 13). See the text for a description of bands IA+II, IA, and II. Polyacrylamide (6%) gel was used. (B) A total of 10^6 cpm of ^32P-labeled PEBP1 probe (the EcoRI-HindIII fragment of pUC13, containing a single PEBP1 binding site; specific activity, 8.0 × 10^7 cpm/µg) was incubated with 2.0 µg of COP-5 cell nuclear extract protein in the presence of poly(dI-dC). The following oligonucleotides were added to the reaction mixture as competitors: none (lanes 1 and 9), WA (lane 2), M1A (lane 3), M2A (lane 4), M3A (lane 5), M4A (lane 6), the EcoRI-HindIII fragment of pUC13 containing a single PEBP1 binding site homologous to the probe (lane 7), a trimer of the PEBP1 binding site (lane 8), WA (lanes 10 to 13), a trimer of WEI (lanes 14 to 17), and a trimer of MEI (lanes 18 to 21). The amount of competitor DNA relative to that of the PEBP1 probe was (in fold molar excess) 200 (lanes 2 to 8), 5 (lanes 10, 14, and 18), 25 (lanes 11, 15, and 19), 125 (lanes 12, 16, and 20), or 625 (lanes 13, 17, and 21). See the text for a description of band IA. Polyacrylamide (6%) gel was used.

Operated by M3A probe are shown (Fig. 6A, lane 1). The WA oligonucleotide competed with the probe in the formation of all three bands in a dose-dependent manner (lanes 2 to 5), while a trimer of WEI (lanes 6 to 9) or MEI (lanes 10 to 13) did not inhibit the formation of band IA or any other band.

A similar competition experiment was carried out by using the ^32P-labeled oligonucleotide representing the PEBP1 binding site as a probe (Fig. 6B). The probe revealed only one major band (IA) with the COP-5 cell nuclear extract (lane 1). This band was confirmed to represent PEBP1, since the addition to the binding reaction of excess of nonlabeled WA, M2A, and M3A (lanes 2, 4, and 5, respectively) but not M1A and M4A (lanes 3 and 6, respectively) oligonucleotides reduced band formation. As expected, a monomer or a tandem trimer of the PEBP1 binding site competed with the probe strongly in the formation of band IA (lanes 7 and 8, respectively). Nonlabeled WA oligonucleotide containing a single PEBP1 binding site prevented the formation of band IA in a dose-dependent manner (lanes 10 to 13), while a trimer of element I, irrespective of the presence or the absence of mutations, did not to any appreciable levels (lanes 18 to 21 and 14 to 17, respectively).

With these results, we conclude that PEBP1 does not bind either to WEI or to MEI even when these sequences are multimerized and, therefore, PEBP1 would have no contribution to the activity of WEI and the trimer of WEI in activating Py DNA replication.
Figure 7 shows the relative positions of the binding sites of PEBP1, PEBP2, and PEBP5 determined in our laboratory. PEBP5 and PEBP1 share at least 5 bp for recognition.

Comparison between the PEBP5 and PEA3 binding sites. Martin et al. detected PEA3 by using the PEA3 oligonucleotide shown in Fig. 8A as a probe, and they proposed that the PEA3 recognition sequence is the hexanucleotide sequence 5′-AGGAAG-3′ (20). They also found another PEA3 recognition sequence overlapping with the B core (Fig. 1) of the Py enhancer (Fig. 8A). Binding of PEBP5 to WEI oligonucleotide was not competed with appreciably by a trimer of the 5′-AGGAAG-3′ sequence (Fig. 8B, lanes 4 and 5), while a trimer of WEI was a very efficient competitor (lanes 2 and 3), as shown above (Fig. 3 and 5B). The oligonucleotide M1A, which contains a monomer of the intact PEBP5 site was an efficient competitor of binding (lanes 10 and 11), while the B-PEA3 oligonucleotide, 5′-TGAAGAGGAAGCTCAAGAA-3′, which spans the second PEA3 binding site shown in Fig. 8A was not (Fig. 8B, lanes 8 and 9). The PEA3 oligonucleotide, 5′-TCGAGAGCATTTGCA-3′, was a weak competitor of our probe (lanes 6 and 7). The underlined sequences are included in the PEBP5 recognition sequence. These results revealed (i) that PEBP5 does not bind to the hexanucleotide 5′-AGGAAG-3′, (ii) that PEBP5 does not bind to the second PEA3 site overlapping with the B core, and (iii) that the PEA3 oligonucleotide containing 8 of 11 bases of PEBP5-binding sequence binds PEBP5 weakly, as in the case of the M4A oligonucleotide (Fig. 5B, lane 15). In other words, results suggest that the binding of PEBP5 weakens when the binding sites is shortened from 5′-CAGGAAGTTGAC-3′ to 5′-CAGGAAATG-3′ and is abolished when the sequence is further shortened to 5′-AGGAAG-3′. Possible relationships between PEA3 and PEBP5 will be discussed below.

Response of PEBP5 to TPA. Since element I is a target of stimulation by TPA (43, 48), the effect of TPA on binding of PEBP5 to element I was examined (Fig. 9). In this particular experiment, NIH 3T3 cells instead of COP-5 cells were used, since COP-5 is a transformed cell line which does not respond well to growth factors. After serum starvation for 48 h, NIH 3T3 cells were treated with TPA for 2 h. A mobility shift assay with WEI as a probe revealed that the nuclear extract of TPA-treated NIH 3T3 cells contained more PEBP5 binding activity, represented by band IB, than that of serum-starved cells (Fig. 9, lanes 2 and 1, respectively). As a control, we measured the level of nuclear factor which bound to another portion of the polyomavirus enhancer (nt 5128 to 5159) and found that the intensity of the band (arrowhead) was not affected by TPA treatment (lanes 3 and 4). The results (Fig. 9) revealed that TPA enhances PEBP5 site DNA-binding activity.

PEBP5 in various cell types. The A core of Py enhancer is functional in undifferentiated F9 cells. In accordance with this is the observation that both PEBP1 (PEA1) (17) and PEBP2 (PEA2) (9, 17), presumed to be positive regulators, are undetectable in F9 cells. It was of interest to examine, therefore, whether PEBP5 would be present in undifferentiated F9 cells.

The nuclear extract from F9 cells induced to differentiate by retinoic acid (dF9) contained PEBP5, which was represented by band IB, formed with WA, M1A, and M2A probes (Fig. 10, lanes 6, 7, and 8) but not with M3A nor M4A probes (lanes 9 and 10). This IB band was hardly detectable in the undifferentiated F9 cell extract (lanes 1 to 3). In addition to PEBP5, PEBP1 and PEBP2 behaved similarly in these two states of F9 cells. PEBP1 represented by band IA was not detectable in F9 cells (lanes 1, 3, and 4) but became detectable in dF9 cells (lanes 6, 8, and 9). Likewise, PEBP2 represented by band II was detectable in dF9 cells (lanes 6, 7, 9, and 10) but was absent in their undifferentiated counterparts (lanes 1, 2, 4, and 5). Under these conditions, the band (IA+II) seen in Fig. 5C and 6A was not clearly detected. We interpreted this to mean that the amounts of PEBP1 and PEBP2 able to form detectable amounts of (IA+II) band under the conditions used were much lower in dF9 than in COP-5 cells.

NIH 3T3 cells contained a relatively small amount of PEBP5, while they were shown previously to express a significant amount of PEBP1 (37). In contrast, several lymphoid cell lines, namely, P388D1 (macrophage cell line), L1210 (B-cell line), and EL4 (T-cell line), contained relatively large amounts of PEBP5, as represented by band IB, but contained no detectable PEBP1 (Fig. 11). In the experiments shown in Fig. 10 and 11, only poly(di-dC) was included in the binding reaction. A trophoblast cell line was also found to contain a relatively high amount of PEBP5 (data not shown). The cell type-dependent expression of PEBP5 observed here suggests its differential regulatory role in different tissues.

DISCUSSION

In the present study, we demonstrated that element I is an important subelement of the A core of the Py enhancer and has the ability to activate Py DNA replication by itself. A nuclear factor, PEBPS, interacting with element I was identified. PEBPS is most likely responsible for the element 1 function that we observed in this as well as in previous studies (48), since PEBP5 increases its DNA-binding activity after TPA treatment. We determined the precise recognition sequence of PEBPS and identified nucleotide residues which are in contact with the factor. PEBP5 was found to be distinct from PEBP1, although their recognition sequences overlap considerably and their expression is regulated similarly in the differentiation of F9 cells.

The replication-activating function of element I alone was rather weak, especially when used as a monomer (Fig. 2B). However, the activity of element I was very significant in the context of the intact A core. First, no other motif in the A core, when tested alone, exerted stronger activity than element I, at least in COP-5 cells (28). Therefore, element I is a major subelement of the A core. Second, element I sequences functioned synergistically with each other or with other motifs. An example of a synergistic effect of element I with other motifs can be recognized by comparing the activities of M3A and WA. The M3A oligonucleotide contained single copies each of the intact PEBP1 and PEBP2 sites and of the mutated PEBP5 site, whereas the WA construct contained single copies each of all three intact sites. This WA construct activated replication at a fourfold higher level than M3A, that is, at a level greater than the sum.
FIG. 8. Comparison of PEBP5 and PEA3 binding sites. (A) Nucleotide sequences of the hexanucleotide and the oligonucleotides PEA3 and B-PEA3 are compared with that of the PEBP5 binding site. The nucleotide numbers of the Py enhancer are indicated. (B) A total of $10^6$ cpm of $^{32}$P-labeled WEI probe (specific activity, $5 \times 10^7$ cpm/μg) was incubated with 4.6 μg of COP-5 cell nuclear extract protein in the presence of poly(dI-dC) and salmon sperm DNA. Oligonucleotides were added to the reaction mixture as competitors, as shown above the lanes. Abbreviations: (WEI)$_3$, a trimer of WEI; (hexanucleotide)$_3$, a trimer of hexanucleotide. The amount of competitor DNA relative to that of WEI probe was (in fold molar excess) 50 (lanes 2, 4, 6, 8, and 10) or 500 (lanes 3, 5, 7, 9, and 11). The band IB represents PEBP5. Polyacrylamide (7%) gel was used.
of the activities of element I and M3A (Fig. 2B, lanes 2, 3, and 6). When WA was dimerized, the level attained was only three- to fivefold less than that of the whole enhancer. The whole enhancer stimulated replication some 1,000-fold above the basal level (Fig. 2A). This hierarchical organization of the Py enhancer is similar to what has been observed with simian virus 40 enhancer for the transcriptional activating function (29). Therefore, we conclude that element I is a major functional unit in the A core as a replication-activating element.

Figure 9. PEBP5 site DNA-binding activity after TPA treatment of cells. NIH 3T3 cells were plated at \( 5 \times 10^5 \) cells per 10-cm dish and incubated for 2 days when the cells became confluent. The medium was then replaced with fresh Dulbecco modified Eagle medium containing 0.5% calf serum and incubated for an additional 48 h. TPA was added to the medium at 100 ng/ml, and cells were further incubated for 120 min before being harvested. The nuclear extract prepared from TPA-treated (lanes 2 and 4) or untreated (lanes 1 and 3) cells was processed for a mobility shift assay. The probes used (10^6 cpm; specific activity, \( 5 \times 10^7 \) cpm/\( \mu \)g) contained WE1 (lanes 1 and 2) or the sequence spanning the Py enhancer from nt 5128 to 5159 (lanes 3 and 4). A total of 2.4 (lanes 1 and 2) or 3.6 (lanes 3 and 4) \( \mu \)g of protein from the extract was used. Poly(dI-dC) and salmon sperm DNA were added in the binding reaction as carriers, and 7% polyacrylamide gel was used. The band IB represents PEBP5. As a control, the level of nuclear factor which bound to another portion of the polyomavirus enhancer (nt 5128 to 5159) was also measured (arrowhead).

Figure 10. Mobility shift assay with F9 or dF9 nuclear extracts. A total of 10^6 cpm of each of the 32P-labeled probes was incubated with 8.0 \( \mu \)g of F9 or dF9 cell nuclear extract protein (shown above each lane) in the presence of poly(dI-dC). Specific activities of all the probes were almost the same (ca. 10^7 cpm/\( \mu \)g). See the text for a description of bands IA, IB, and II. Polyacrylamide (6%) gel was used.

replication were found to be equivalent to the transcription factors CCAAT box-binding protein (CTF) and octamer-binding protein 1 (OTF-1), respectively (16, 30, 31). Thus, PEBP5 could be a member of this class of protein.

The existence of a factor which interacts with element I was suggested by the presence of a DNase I hypersensitive site at nt 5136 (HS-5136A3, the numbering system based on the Py A strain [4], corresponding to nt 5111, as described for the A2 strain [38] which we used in the present study [20]). HS-5136A3 was shown not to be due to the binding of PEA1 or PEA2 (PEBP1 or PEBP2) but to a new factor designated PEA3. A mobility shift assay with the oligonucleotide probe 5'-TCGAGCAGGAAGTTCGA-3' revealed a weak binding activity in the 3T6 cell nuclear extract. The underlined sequence, AGGAAG, was suggested to be the PEA3 binding site (20). We compared the PEA3 binding site with the PEBP5 binding site determined in the present study. The result revealed that PEBP5 binds only weakly to a part of element I sequence, 5'-CACGAGAG-3', which is present in the PEA3 oligonucleotide and is no longer able to form a stable complex with a further-shortened sequence, 5'-AGGA-3'. These results suggest either one of the following two possibilities: (i) PEA3 and PEBP5 are distinct, or (ii) PEA3 and PEBP5 are identical but the recognition sequence suggested for PEA3 binding is inappropriate. The second possibility seems to be the case, since the relative mobilities of PEBP1 and PEBP5 (Fig. 5C) are similar to those of PEA1 and PEA3 (20). Furthermore, element I was found to be responsive to TPA and the activated Ha-ras oncogene (48), and the DNA binding activity of PEBP5 was enhanced by TPA, while PEA3 was reported to behave similarly (43).
Since multiple factors are known to interact with a single sequence motif, more rigorous examination would be necessary to definitively determine the relationship between PEA3 and PEBP5.

A nuclear factor, EF-1A, which binds to the core element of adenovirus type 5 E1A enhancer, has been identified in human and rodent cells (2). EF-1A binds 10 times as strongly when it binds to two sites than to a single one. EF-1A does not seem to bind significantly to a single copy of the sequence, which is very similar to prototype element I, and is located at nt -300 of the E1A gene. The Py enhancer contains a single copy of element I, and we detected PEBP5 relatively easily with the probe containing a single copy of element I. According to Bruder and Hearing (2), the Py enhancer competes for binding of EF-1A to the DNA probe containing two sites, but only about fivefold less efficiently than the homologous competitor. Further studies are necessary to definitively determine the relationship between EF-1A and PEBP5.

There are two more factors, E4TF1 and IRF-1, which recognize sequence motifs similar to the V element. However, E4TF1, which binds to the 5’-CGGAAGTGCA-3’ sequence in the promoter of the adenovirus type 5 E4 gene (47), and interferon regulatory factor 1, IRF-1, which binds to the hexamer sequence (5’-AAGTGCA-3’) of the β-interferon gene promoter (8, 24), appeared to be distinct from PEBP5, on the basis of competition studies in mobility shift assays (data not shown).

It was shown previously that the function of the PEBP5 binding site on transcription was enhanced by TPA and by the activated Ha-ras oncogene (48). We showed, in the present study, that TPA increases the DNA-binding activity at the PEBP5 site, suggesting that PEBP5 is a receiver of growth signals initiated by TPA, an activator of protein kinase C. More recently, the element I binding protein PEA3 (possibly equivalent to PEBP5) has been shown to be stimulated by several other nonnuclear oncogenes (43). In this sense PEBP5 could be as equally important a regulatory factor as AP-1, which is a well known TPA-responsive factor. AP-1 is a protein complex formed by the products of c-jun and c-fos proto-oncogenes (3, 7, 34, 36). PEBP5 therefore requires rigorous characterization in relation to growth control and carcinogenesis.

The A core is nonfunctional as a transcriptional enhancer in F9 cells. We and others have identified three regulatory factors whose binding sites cover the entire length of the A core (Fig. 7) (15, 20, 33, 37). All of these factors, PEBP1, PEBP2, and PEBP5, were absent or present in limited amounts in F9 cells and became easily detectable after F9 cells were induced to differentiate. PEBP1 is a member of the AP-1 family and is a positive factor. Similarly, PEBP5 is likely to be a positive factor, since TPA increases its DNA-binding activity and transcriptional and replicational activity simultaneously (28, 48). Therefore, at least part of the reason why the A core is inert in F9 cells appears to be the shortage of positive regulators, PEBP1 and PEBP5. In the case of PEBP2 (PEA2), it has been reported that the factor is a negative regulator (44). However, analysis of the PEBP2 binding site revealed a more complicated mechanism for its regulation. The results are fully described elsewhere (9).

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