Purification of a Mouse Nuclear Factor That Binds to both the A and B Cores of the Polyomavirus Enhancer

YUSUKE KAMACHI,¹ EIKO OGAWA,² MAKI ASANO,² SEIKO ISHIDA,² YOTA MURAKAMI,²
MASANOBU SATAKE,² YOSHIKI ITO,² AND KATSUYA SHIGESADA^{1*}

*Departments of Biochemistry¹ and Viral Oncology,² Institute for
Virus Research, Kyoto University, Kyoto 606, Japan*

Received 2 April 1990/Accepted 10 July 1990

We have previously identified a protein factor, PEBP2 (polyomavirus enhancer-binding protein), in the nuclear extract from mouse NIH 3T3 cells which binds to the sequence motif, PEA2, located within the polyomavirus enhancer A element. Upon cellular transformation with activated oncogene *c-Ha-ras*, this factor frequently undergoes drastic molecular modifications into an altered form having a considerably reduced molecular size. In this study, the altered form, PEBP3, was purified to near homogeneity. The purified PEBP3 comprised two sets of families of polypeptides, α -1 to α -4 and β -1 to β -2, which were 30 to 35 kilodaltons and 20 to 25 kilodaltons in size, respectively. Both kinds of polypeptides possessed DNA-binding activities with exactly the same sequence specificity. Individual α or β polypeptides complexed with DNA showed faster gel mobilities than did PEBP3. However, the original gel retardation pattern was restored when α and β polypeptides were mixed together in any arbitrary pair. These observations along with the results of UV- and chemical-cross-linking studies led us to conclude that PEBP3 is a heterodimer of α and β subunits, potentially having a divalent DNA-binding activity. Furthermore, PEBP3 was found to bind a second, hitherto-unnoticed site of the polyomavirus enhancer that is located within the B element and coincides with the sequence previously known as the simian virus 40 enhancer core homology. From comparison of this and the original binding sites, the consensus sequence for PEBP3 was defined to be PuACCPuCA. These findings provided new insights into the biological significance of PEBP3 and PEBP2.

Polyomavirus (Py) provides a useful model to study cell-type-specific control of eucaryotic gene expression. Both the replication and transcription of this virus are dependent on its enhancer sequence (see Fig. 1) located between the *Bcl*I site (nucleotide [nt] 5021) and the origin-proximal *Pvu*II site (nt 5265) (10, 19, 33, 50). This enhancer is inactive in embryonal carcinoma cells, such as F9 cells, and becomes active after differentiation of these cells (11, 24, 46, 47). Its activity is also modulated by various viral and cellular oncogenes, either positively or negatively (5, 18, 44, 50, 52, 54).

The 244-base-pair (bp) Py enhancer can be divided into four subdomains, A, B, C, and D (51). Of these, elements A and B, respectively localized to nt 5107 to 5130 and nt 5179 to 5214, retain minimum sufficient potential to activate both replication and transcription, while elements C and D serve to augment these functions in auxiliary manners (19, 51). Studies in other laboratories identified three functional elements, I, II, and III, for transcriptional activation (34) or α and β cores for replication activation (35). The elements II and III or the α and β cores roughly correspond to the A and B elements, respectively. Subsequent studies in this and other laboratories have shown that the A element interacts with at least three nuclear factors, designated PEA1, PEA2, and PEA3 (42) or PEBP1, PEBP2, and PEBP5 (44, 56; M. Asano, Y. Murakami, K. Furukawa, Y. Yamaguchi-Iwai, M. Satake, and Y. Ito, submitted for publication). PEBP1 (44, 45) or PEA1 (42) binds to the sequence TGACTAA (nt 5113 to 5121), which resembles the consensus recognition sequence of the transcription activation protein, AP1, isolated from HeLa cells (30). Like the AP1 motif (2, 30), the

PEA1 site is responsive to transcriptional activation by a tumor promoter, 12-tetradecanoylphorbol-13-acetate (21, 56) or oncogene *Ha-ras* (44, 54). In addition, the product of proto-oncogene *c-jun*, a known member of the AP1 family (1, 4), was shown to bind to the PEBP1 site (20). Thus, PEBP1 is thought to be a murine counterpart of AP1. PEBP5 (56) or PEA3 (31) recognizes the sequence which partly overlaps the PEBP1 site and is homologous to the adenovirus E1A enhancer core called element I (17). The PEBP5 site can also act as a transcriptional enhancer, being responsive to 12-tetradecanoylphorbol-13-acetate or transiently expressed *Ha-ras* oncogene (53, 56). Interestingly, the PEBP1 and PEBP5 sites function synergistically rather than competitively, despite their overlapping locations (36, 56). Since these factors are absent in F9 cells and become detectable only after cell differentiation (Asano et al., submitted), they are likely to be responsible, at least partly, for the differentiation-dependent activation of the Py enhancer noted above (see below for possible involvement of PEBP3 in this activation).

Thus far, less clear is the role of the third factor, PEBP2 (44) or PEA2 (42), which binds to the sequence GACCGC (nt 5123 to 5128). However, growing evidence suggests its biological importance. First, PEBP2 is a differentiation-specific factor, which is detectable by gel retardation assay in fibroblasts (44) and retinoic acid-induced F9 cells (12) but not in undifferentiated F9 cells (12). Second, the PEBP2-binding activity detected in the nuclear extract from *Ha-ras*-transformed NIH 3T3 cells (44) shows a new retarded band (indicated as D in Fig. 1B) migrating in gels much faster than the one originally present in normal NIH 3T3 cells (see Fig. 1B, indicated as C). Thus, PEBP2 appears to undergo molecular and functional modulations in association with cellular oncogenesis. Third, the PEBP2 site has profound

* Corresponding author.

influence on the overall function of the Py enhancer. For instance, the capacity of the A element to activate replication was drastically decreased when this site was impaired by deletions (51) or base substitutions (36). Furthermore, the PEBP2-binding site was found to overlap the recognition sequence (nt 5119 to 5138) of another nuclear factor, PEBP4, which was recently identified in this laboratory as a factor responsible for the F9 cell-specific repression of the Py enhancer activity (12). Since PEBP4 is also detectable in retinoic acid-treated F9 cells and fibroblasts along with PEBP2, these factors could functionally antagonize each other in these cells. On the other hand, PEBP2 and PEBP1 can simultaneously bind to their adjacent recognition sites, thus opening the possibility of their functional cooperation (31, 42, 44). Through such interplay, PEBP2 may positively contribute to the derepression of the Py enhancer activity upon differentiation (12). However, Wasylyk et al. (52) recently presented an opposite view that the PEA2 (PEBP2) site would serve as a negative transcriptional regulator because it seemed to inhibit the enhancer activity due to the PEA1 (PEBP1) motif as measured with mouse L cells and F9 cells as hosts. Presumably, the apparent regulatory influence of the PEBP2 site could depend on its context within the sequence tested as well as the type of host cells employed.

To gain further insight into the molecular nature and regulatory role of the PEBP2-binding factor(s), we set out to purify it from mouse fibroblasts. As a first step, this paper describes purification and properties of its variant form as detected in Ha-ras-transformed NIH 3T3 cells. The results reveal that this factor is a heterodimer of two different groups of polypeptides and that it also binds to a hitherto-unnoticed site within the B element almost as strongly as to the original PEBP2 site. This implies that the factor should have broader significance in control of the Py enhancer function than was previously envisioned.

MATERIALS AND METHODS

Cells. NIH 3T3 cells transformed by activated Ha-ras oncogene and their tumor derivative induced in nude mice were grown as described elsewhere (44).

DNA affinity column. Complementary deoxyoligonucleotides representing the PEA2 sequence (nt 5121 through 5130) with a 4-nt extension were prepared in a DNA synthesizer (Applied Biosystems, Inc., model 380B): 5'-GATCCT GACCGCAG and 5'-GATCCTGCGGTCAG. Similarly, mutant oligonucleotides containing 2-bp changes (underlined bases) were 5'-GATCCTCACGGCAG and 5'-GATCCTGC CGTGAG. These oligonucleotide pairs were annealed and concatenated with T4 DNA ligase and coupled to CNBr-activated Sepharose CL-2B (Pharmacia) as described previously (23). The amounts of coupled DNA were approximately 24 and 14 µg per ml of gel matrix for the wild-type and mutant oligonucleotides, respectively.

Purification procedure. Nuclear extracts were prepared from Ha-ras-transformed NIH 3T3 cells or their tumor derivative as previously described (44). PEBP3 activity was monitored by gel retardation assay as described elsewhere (44). The nuclear extract (ca. 100 mg of protein) was fractionated with ammonium sulfate (55% saturation). Precipitated protein was dialyzed to buffer D (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride) containing 0.1 M KCl and loaded onto a 10-ml heparin-Sepharose column (Pharmacia) equilibrated with the same buffer. After

washing with buffer D-0.1 M KCl, protein was eluted with step gradients of KCl in buffer D (0.2, 0.35, and 0.5 M). Active fractions (0.35 M KCl) were pooled, concentrated, and subjected to gel filtration on a TSK G3000SW column (7.8 mm by 60 cm, Tosoh) in 0.2 M sodium phosphate buffer (pH 6.8) containing 10% glycerol and 1 mM DTT. Active fractions were pooled, dialyzed to buffer Z (20 mM HEPES-KOH, pH 7.6, 20% glycerol, 0.1% Nonidet P-40, 0.2 mM EDTA, 1 mM DTT) containing 50 mM KCl, incubated with poly(dI-dC) (Pharmacia, 1.5 µg/ml), and loaded onto a 2-ml mutant-oligonucleotide-Sepharose column equilibrated with the same buffer. After washing with buffer Z-50 mM KCl, PEBP3 activity was eluted with buffer Z-0.25 M KCl. This fraction was diluted to 0.1 M in KCl with buffer Z, incubated with poly(dI-dC) (1 µg/ml), and loaded onto a 1-ml wild-type-oligonucleotide-Sepharose column equilibrated with buffer Z-0.1 M KCl. The column was successively washed with buffer Z containing 0.1 and 0.4 M KCl, and PEBP3 activity was eluted with buffer Z-1 M KCl. Active fractions were diluted to 0.1 M in KCl with buffer Z and rechromatographed on a 0.2-ml wild-type-oligonucleotide-Sepharose column as described previously.

Renaturation of protein. The affinity-purified PEBP3 was electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gels (26), and each protein component resolved was renatured essentially as described previously (15). Briefly, protein bands were visualized by staining with CuCl₂ (29), excised separately, and destained by successive incubations in 0.25 M Tris hydrochloride (pH 9)-0.25 M EDTA and elution buffer (50 mM Tris base, 50 mM glycine, 0.1% SDS). Protein was electrophoretically eluted from gels, precipitated with 4 volumes of acetone, and taken up in 20 µl of 6 M guanidinium hydrochloride-buffer R (25 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.1 M KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT). The protein thus recovered was placed in a microdialyzer (Pierce Chemical Co.) and allowed to renature by dialysis to buffer R at 4°C overnight.

Gel retardation assay. The 24-bp A core sequence (nt 5107 to 5130) of the Py enhancer (see Fig. 1) was cloned into the *Bam*HI site of pUC13. From the resultant recombinant, the *Hind*III-*Eco*RI segment containing the A core was excised and labeled with ³²P at both ends. The gel retardation assay was performed with this probe as described previously (44). Two kinds of DNA fragments similar to the one described above but altered at the PEBP1 or PEBP2 site (43) were prepared and used in unlabeled forms for binding competition experiments as combined with the gel retardation assay: M1, GTGACTAAC → GTGACAAAG; M2, GACCGC → CACGGC. Standard DNA manipulations were performed as described elsewhere (40).

Methylation interference footprinting. The same A element fragment as that used for the gel retardation assay was asymmetrically labeled with ³²P at the 3' end on either strand. The methylation interference analysis was performed with the labeled probe as described previously (44) except that recovered DNAs were cleaved by G- and A-specific chemical reactions instead of only a G-specific one (32).

DNase I footprinting. The *Hind*III-*Sma*I fragment containing the whole Py enhancer region (nt 5021 to 5265) cloned into the *Bam*HI site of pUC13 and the *Hind*III-*Pvu*II fragment containing the simian virus 40 (SV40) enhancer (nt 5172 to 5243 and 1 to 270) from pSV2-CAT (14) were asymmetrically end labeled with ³²P by filling in their unpaired *Hind*III ends. DNase I footprinting was performed with these probes essentially as described in reference 40, except MgCl₂ and CaCl₂ were spared from the binding buffer and only added at

FIG. 1. Gel retardation assays of nuclear factors binding to the Py enhancer A element. (A) Diagram of the Py enhancer. Shown are the four functional domains, A, B, C, and D (51). The sequence of the early strand of element A (nt 5107 through nt 5130) is shown at the bottom. Boxed regions indicate the minimum recognition sites for PEBP1, PEBP2, and PEBP3, as determined by methylation interference experiments (44). (B) Gel retardation pattern. A ^{32}P -labeled A-element probe was incubated with 5 μg of protein of nuclear extract from the indicated cells and subjected to gel electrophoresis as previously described (44). Lane 1, NIH 3T3 cells; lane 2, Ha-*ras*-transformed NIH 3T3 cells cultured in vitro; lane 3, tumors generated from Ha-*ras*-transformed NIH 3T3 cells in nude mice. The positions of major protein-DNA complexes (A to D) and the free probe DNA (F) are marked.

FIG. 2. Molecular size analysis of PEBP3. (A) Gel filtration profile of native PEBP3 activity. Fractions eluted from the TSK G3000SW column were subjected to a gel retardation assay. The leftmost lane represents the original sample before loading. The arrows with numbers indicate the positions and molecular sizes (in kilodaltons) of marker proteins. D and F indicate the PEBP3-probe DNA complex and the free probe DNA, respectively. (B) SDS-polyacrylamide gel electrophoresis of affinity-purified PEBP3. Fractions eluted from the second wild-type DNA-Sepharose column at 1 M KCl were run on an SDS-polyacrylamide gel (12.5%) and stained with silver (lanes 1 to 4 in the order of elution). One-twentieth of each fraction derived from 100 mg of protein of nuclear extract was loaded on each lane. Lane M, Marker proteins with their molecular sizes indicated in kilodaltons on the left. The marker proteins were applied at 20 to 30 ng each. Two groups of clustered bands detected in correlation with the PEBP3 activity (lanes 2 and 3) are designated α -1 to α -4 and β -1 and β -2. Additional bands seen in all lanes are marked as X.

the stage of DNase I digestion in reduced amounts (final concentration, 0.5 mM MgCl₂ and 0.2 mM CaCl₂). These modifications were necessary because the DNA-binding activity of PEBP3 was rather sensitive to those divalent cations.

UV and chemical cross-linking. Two deoxyribo-oligonucleotides corresponding to nt 5174 to 5185 (5'-CCCTCCA GAGGG, early strand) and nt 5174 to 5205 (5'-CCTCTTG CAAAACCACTGCCCTCTGGAGGG, late strand) of the Py enhancer were synthesized and annealed together, and the missing part of the early strand was filled in by T4 DNA polymerase reaction in the presence of [α -³²P]dATP, bromo-dUTP, dGTP, and dCTP. UV cross-linking was performed with this probe by the method previously described (8), with some modifications. The binding-reaction mixture (total volume, 10 μ l) contained 20 mM triethanolamine-HCl buffer, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 4% (wt/vol) Ficoll (Pharmacia), 0.1 ng of the probe DNA, 0.1 μ g of poly(dI-dC), and an appropriate amount of purified PEBP3 or its isolated subunit. The mixture (placed in a short, 0.5-ml propylene tube) was incubated at 25°C for 30 min, irradiated with a short-wave UV transilluminator (model TS-15, UVP Inc.) from 5 cm above for 60 min at 4°C, and then subjected to electrophoresis in 12.5% polyacrylamide gels containing 0.1% SDS. To detect intersubunit contacts, cross-linking with glutaraldehyde (28) was performed as follows. The UV-irradiated reaction mixture, processed as described above, except with an omission of DTT from the buffer, was supplemented with 1.1 μ l of a glutaraldehyde solution (final concentration, 0.01 to 0.1% [vol/vol]) and incubated for another 60 min at 25°C. The reaction was stopped by adding 1.2 μ l of 1 M Tris hydrochloride buffer, pH 8.0, and the products were analyzed by SDS-polyacrylamide gel electrophoresis as described above.

Replication assay. Plasmids for the DNA replication test, pPyOICAT and its derivative [pPy(BPP)OICAT] containing the whole Py enhancer (nt 5021 to 5265), were described previously (36). Double point mutations designated MA and MB were introduced by oligonucleotide-mediated site-directed mutagenesis (39) into the respective PEBP2/PEBP3-binding sites within the A and B enhancer elements, and either or both of these mutations were substituted into pPy(BPP)OICAT (see Fig. 8B). The resultant derivatives were transfected into COP5 cells (49) together with the control plasmid (pUC13) by the DEAE-dextran method exactly as described previously (36). After 24 h of transfection, replicated plasmid DNA was extracted, separated by agarose gel electrophoresis after digestion with *DpnI* and *HindIII*, and detected by hybridization with ³²P-labeled DNA probes as previously described (36).

RESULTS

Nomenclature of protein factors. Initially, we named the factors which gave rise to DNA-protein complexes B, C, and D (Fig. 1B) as B, C, and D factors, respectively (44). The band A in Fig. 1B represents a ternary complex between B and C factors and the DNA probe (44). Since the target of the DNA-binding factor in question is not limited to the PEA2 site, as will be shown, we propose a new series name, PEBP (polyomavirus enhancer-binding protein), for this and related factors (56). PEBP2 is the PEA2-binding factor originally identified in NIH 3T3 cells; PEBP3 is the counterpart detected in Ha-ras-transformed cells. Similarly, PEBP1 will be assigned to the former B factor or PEA1 (42).

Purification of PEBP3. As reported previously (44), the

FIG. 3. DNA-binding activities and complex formation of the α and β polypeptides. (A) Polypeptides α -1 to α -4, β -1, β -2, and X as detected in Fig. 2B were separately eluted from SDS-polyacrylamide gel, renatured, and subjected to gel retardation assay either individually or in combination as specified on the top of each lane. The amounts of each polypeptide used per lane were 2 μ l for all species when tested alone and 0.5 and 1 μ l for α and β polypeptides when tested as mixtures. Lane D, The original affinity-purified PEBP3 (0.5 μ l). (B) DNA-binding specificities of α and β polypeptides compared with that of the original PEBP3. The affinity-purified PEBP3 and mixtures of renatured α and β polypeptides in each group were bound to the A-element probe in the presence of a 200-fold molar excess of the unlabeled competitor DNA indicated above each lane and subjected to gel retardation assay as for panel A. W, Wild-type A element; M1, mutant A element altered in the PEBP1 site (GTGACTAAC $\rightarrow$ GTGACAAAG); M2, mutant A element altered in the PEBP2 site (GACCGC $\rightarrow$ CACGGC); D, PEBP3-probe DNA complex; F, free probe DNA.

PEBP3 activity was detected in abundance in the nuclear extract from Ha-ras-transformed NIH 3T3 cells, either cultivated in vitro or grown into tumors in nude mice (Fig. 1B, band D in lanes 2 and 3). Since the tumor tissue was easier to obtain in bulk, we routinely used it as a source of PEBP3 in the following trial of purification.

The purification procedure involved four chromatographic steps: heparin-Sepharose chromatography, high-performance

FIG. 4. Fine mapping of the recognition sites for PEBP3 and the α and β subunits. Methylation interference experiments were performed with the affinity-purified PEBP3 and renatured polypeptides and with the A-element probe as described in Materials and Methods. The probe was asymmetrically end labeled on the early strand (A) or on the late strand (B). For each protein sample tested, three DNA products were run in a set: N, the original probe; C, DNA bound to protein; F, free DNA not complexed with protein. The minimum PEBP3 recognition sequence is shown on the right. Triangles denote purine residues, whose methylation interferes strongly (▲) or weakly (◁) with binding of PEBP3 or its subcomponents.

liquid chromatography gel filtration on a TSK G3000SW column, and chromatography on specific DNA affinity columns with the wild-type sequences and with the mutated PEA2 sequences. At the gel filtration step, the PEBP3 activity was eluted in a broad peak over a molecular size range of 30 to 60 kilodaltons (kDa) (Fig. 2A). This suggested that PEBP3 might comprise multiple polypeptides of heterogeneous sizes or a dissociable complex of a smaller subunit(s). In fact, the final preparation obtained after two passages through the wild-type DNA column revealed two groups of clustered polypeptide bands corresponding to 30 to 35 kDa (α -1 to α -4) and 20 to 25 kDa (β -1 and β -2) on SDS-polyacrylamide gel electrophoresis (Fig. 2B, lanes 2 and 3). The two groups of polypeptides showed distinctive colors upon silver staining (brown with α bands versus blue with β bands), suggesting that they were chemically homologous within each group but different between groups. The few additional bands at 40 to 50 kDa are regarded as artifacts of silver staining because they commonly appeared in most fractions (Fig. 2B, band X).

Judging from the intensities of the α and β bands with those of marker proteins, the yields of these polypeptides were estimated to be 0.5 to 1 μ g per group from the nuclear extract containing 100 mg of protein. The overall recovery of PEBP3 was approximately 10% over the starting nuclear extract.

Identification of DNA-binding polypeptides. To determine which of the multiple polypeptide species detected in the final PEBP3 preparation were responsible for DNA-binding activity, we undertook to renature the individual polypeptides extracted from the gel and subjected them to gel retardation assay. Surprisingly, each of the α and β polypeptides showed definite DNA-binding activity (Fig. 3A). No such activity was detected in the 40- to 50-kDa polypeptides (Fig. 3A, lane X). The DNA-protein complexes formed with

individual α or β polypeptides migrated faster than the original PEBP3-DNA complex (Fig. 3A). When the α or β polypeptides were mixed within each group, they still gave rise to faster-moving bands, which appeared as though the bands due to individual polypeptides were simply superimposed together. This implies that the members of each group may either have no mutual interactions at all or freely mingle together to form homo- and heteromeric complexes involving a constant number of subunits. In contrast, the α and β polypeptides mixed together (Fig. 3A, lane $\alpha + \beta_{\text{MIX}}$) produced a new band which had virtually the same mobility as the one originally observed with PEBP3. This result indicates that the two groups of polypeptides preferentially associate to generate protein-DNA complexes which are larger than those formed with each polypeptide species alone. Similar complex formation was also observed when individual polypeptides from both groups were mixed in any arbitrary pair (see Fig. 6 for a typical result obtained with the combination of α -3 and β -1). Thus, α and β polypeptides appear to be functionally equivalent within each group but different between groups, showing a striking correlation with their distinct chemical natures as noted in the preceding section. At present, it is unknown whether α and β polypeptides represent distinct members of a protein family or they merely differ from each other by posttranslational modification. Until this question is settled in the future, we would like to use the notation α and β solely to signify that they are functionally distinguishable entities.

Detailed inspection of the gel retardation patterns in Fig. 3A provides further clues to possible protomeric structures of protein-DNA complexes formed with individual and mixed polypeptides. The large difference between the mobilities of the α and β bands may most simply be explained if the individual α subunits are complexed with DNA as a monomer and the $\alpha\beta$ pair is complexed with DNA as a

FIG. 5. UV and glutaraldehyde cross-linking of α and β subunits with DNA and between each other: (A) UV cross-linking of each subunit with DNA. The affinity-purified PEBP3 and renatured individual subunits were bound to bromodeoxyuridine-substituted and ^{32}P -labeled B-element probe, irradiated with UV light, and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The amounts of the protein samples used per lane were 0.5 μl of PEBP3, 2 μl of α -3, and 4 μl of β -2. The positions of molecular size markers (in kilodaltons) and presumed polypeptide-DNA adducts are indicated on the left and right, respectively. Weaker signals were obtained with α and β polypeptides than with PEBP3 because of their poor recoveries from renaturation. Bands corresponding to unbound DNA were cut out from the gel before autoradiography in the particular experiment presented. (B) Double cross-linking of PEBP3-DNA complexes by UV irradiation and glutaraldehyde treatment. PEBP3-DNA adducts obtained as described above were further treated with various concentrations of glutaraldehyde (percent [vol/vol] as indicated above each lane) and then subjected to SDS-polyacrylamide gel electrophoresis. The initial amount of PEBP3 used per lane was 1 μl . The positions of putative α - and β -DNA ternary adducts is indicated on the right. Molecular sizes (in kilodaltons) are indicated on the left. F, Free probe DNA.

heterodimer. Consistent with this notion is the previous observation upon gel filtration analysis that PEBP3 has a broad size distribution, ranging up to 50 to 60 kDa at maximum, which value corresponds well to the combined molecular mass of α and β polypeptides on the average. Curiously, the β bands were visibly more retarded than the α bands and rather close to the $\alpha\beta$ bands, albeit β polypeptides (20 to 25 kDa) were considerably smaller in size than α polypeptides (30 to 35 kDa) and even more so than the putative $\alpha\beta$ heterodimer (50 to 60 kDa, on average). Such an anomaly in the gel mobility could result if β polypeptides form homodimers (40 to 50 kDa).

One may argue that the gel mobility measured under nondenaturing conditions might not directly reflect the molecular mass of a DNA-binding factor because it could be variously affected by the net charge or steric configuration of that factor as well. Nevertheless, we can definitely exclude one particular alternative model that both α and β exist mainly as homodimers by themselves and rearrange to heterodimers when mixed together. If so, the $\alpha\beta$ band should migrate between the α and β bands, in clear contradiction to what was actually observed.

Mode of interactions of α and β subunits with DNA. How do the two kinds of subunits, α and β , participate in the recognition of the PEBP2/PEBP3-binding sequence, and what is the functional significance of their dimerization? To answer these questions, we investigated the detailed DNA-binding specificity of α and β by binding competition and methylation interference experiments. First, mixed polypeptides of each group were incubated with the labeled A-ele-

ment probe in the presence of various nonlabeled DNA fragments and subjected to a gel retardation assay (Fig. 3B). All of the bands generated with α and β , either alone or combined together, were effectively abolished by competitors carrying the intact PEBP2/PEBP3 site (W and M2) but not by the one mutated at 2 bp within that site (M2). Next, the same series of protein preparations as that described above were subjected to methylation interference analysis with the A-element probe terminally labeled on either strand. The resulting footprints (Fig. 4) revealed that α and β polypeptides contact with identical sets of A and G bases (marked by asterisks) on each strand of the probe, regardless of whether they were separate or combined. These results indicate that the α and β polypeptides independently recognize the whole PEBP2/PEBP3-binding sequence in one unit.

To directly confirm the presumptive molecular association of the α and β polypeptides with DNA as well as between these subunits, we further carried out UV-cross-linking studies as follows. As a DNA ligand, we used a 32-bp synthetic DNA fragment encompassing the SV40 homology sequence within the Py enhancer B element (nt 5174 to 5205), which was found to contain an effective PEBP3-binding site as described below. This site is richer in T residues than the original one in the A element and therefore can serve a better target for UV cross-linking upon substitution of those residues with bromodeoxyuridine residues. A ^{32}P -labeled B-element probe was incubated with α or β polypeptide or their heterodimer, irradiated with a short UV light, and then subjected to electrophoresis in SDS-polyacrylamide gels. Upon UV cross-linking, isolated polypep-

FIG. 6. Gel retardation patterns with renatured polypeptides α -3 and β -1 mixed in various ratios. Numbers at the top of each lane indicate the amounts (in microliters) of each polypeptide used. The positions of shifted bands due to individual and complexed polypeptides are indicated on the left. F, Free probe DNA.

tides α -3 and β -2 yielded a few protein-DNA adducts with mobilities corresponding to 30 to 40 kDa and 22 to 28 kDa, respectively, which values were slightly larger than their estimated molecular sizes (Fig. 5A). Similarly, the original PEBP3 showed multiple bands which distributed over a wider range (20 to 40 kDa) and were presumed to represent all subspecies of α and β contained therein. These results suggest that only a monomer of each polypeptide was covalently attached per single DNA ligand, regardless of whether the protein factor in question was monomeric or dimeric. If two polypeptides were simultaneously cross-linked to one DNA ligand, the resultant product should show a larger apparent molecular size corresponding to the total mass of $\alpha\beta$ or $\beta\beta$ dimers (50 to 60 kDa) plus an extra drag due to the DNA ligand. Such slower-moving bands actually became detectable only when the UV-irradiated PEBP3-DNA complexes were further treated with a protein-cross-linking agent, glutaraldehyde (Fig. 5B). This result is consistent with and lends further support to the proposed model that α and β subunits form heterodimers primarily through protein-protein interactions and each of them potentially binds to a single entire recognition site in an independent manner.

Possible action of PEBP3 as a divalent DNA-binding factor. The preceding conclusion in turn predicts that the $\alpha\beta$ heterodimer and probably also the putative $\beta\beta$ homodimer could act as a divalent DNA binding factor. To test this possibility, we carried out a titration experiment, in which a fixed amount of polypeptide α -3 was mixed with increasing amounts of β -1 and subjected to a gel retardation assay with the A-element probe. The α polypeptide was almost completely converted to the $\alpha\beta$ complex in the presence of polypeptide β in excess (Fig. 6). In accord with the above

prediction, the $\alpha\beta$ band contained precisely twice as much radioactivity (2,287 cpm) as the original band (1,147 cpm) when measured at the maximum extent of conversion. It should be noted, however, that this result could also be explained if the $\alpha\beta$ complex had a higher affinity for DNA than the isolated α polypeptide. As another, more straightforward test, we performed a gel retardation assay using a mixture of two DNA probes which were different in length but equally carried a single PEA2 site (the 77-bp A-element probe and a 240-bp fragment containing the A element with an extra pUC13-derived appendage). If the $\alpha\beta$ or $\beta\beta$ dimer could simultaneously bind the two different probes, it should give rise to retarded bands migrating between the control bands produced with the respective probes alone. However, no such intermediate band was detected under any conditions examined (data not shown). It thus seems that the putative complexes bearing two rather long DNA strings, if formed, might not be stable enough to withstand gel electrophoresis or those dimeric factors could not simultaneously interact with two DNA ligands after all. Further work is needed to resolve between these possibilities.

Identification of a second PEBP3-binding site within the B enhancer element. The putative divalent nature of PEBP3 noted above prompted us to ask whether the Py enhancer has target sites for this factor other than the PEA2 site. To this end, we carried out DNase I footprinting experiments using a DNA fragment containing the whole Py enhancer sequence. The resultant footprints revealed two protected regions: one was located at nt 5121 to 5133, just coinciding with the PEA2 site, and the other was at nt 5185 to 5197 within the enhancer element B (Fig. 7A). Judging from the extents of protection in the DNase I footprinting, PEBP3 appeared to have roughly the same, though slightly less, affinity for the B-element site as for the PEA2 site. Upon visual inspection, the protected sequence at the second site turned out to contain a 7-bp motif, AACCACA (nt 5195 to 5189 on the late strand), that closely resembles the original PEA2 sequence, GACCGCA (nt 5123 to 5129 on the early strand) (Fig. 7C). Additional evidence which suggests that the newly recognized motif can actually serve as an effective target for PEBP3 is described below.

We then asked whether PEBP3 binds to the two sites in a cooperative manner. To this end, we compared the binding of PEBP3 to two different DNA fragments: one was the whole Py enhancer sequence carrying both the A element and the B element as used above, and the other contained only the B element. The concentration of PEBP3 required for a half-maximal protection of the B-element site from DNase I cleavage was virtually the same for both DNA fragments (data not shown). Thus, there seemed to be no functional interaction in the binding of this factor to the A- and B-element sites on the whole Py enhancer, at least under the experimental conditions tested.

Further evaluation of the DNA-binding specificity of PEBP3. The site newly detected within the B element closely overlaps the sequence motif previously noted for its homology to the SV40 enhancer core, TGTGGA/TA/TA/T (34, 55, 58). The SV40 core has been shown to bind various transcription factors, such as AP-3 (7), EBP20 (22), PEB1 (41), and TEF-2 (9). To see whether PEBP3 could also bind to the original SV40 core, we carried out DNase I footprinting with the whole SV40 enhancer sequence (Fig. 7B). However, no binding to the expected site present within each of the two 72-bp repeats was observed, indicating that PEBP3 is clearly different from the factors mentioned above. Instead, a weak binding site, about one order less efficient than the PEA2

FIG. 7. DNase I footprinting of PEBP3 binding to the enhancer regions of Py and SV40. DNase I footprints were generated with indicated probe DNAs with the affinity-purified PEBP3 essentially as described elsewhere (40). (A) The Py enhancer; (B) the SV40 enhancer. Lanes 1, G+A cleavage products; lanes 3 to 5, footprints obtained with increasing amounts of PEBP3 as indicated (0.1, 0.3, and 1 μ l [A] and 1, 3, and 10 μ l [B], respectively); lanes 2, control without added PEBP3. Nucleotide positions are shown on the left. Vertical lines on the right indicate the functional regions A, B, C, and D for the Py enhancer (51) or the 21- and 72-bp repeats for the SV40 enhancer. Interrupted lines indicate protected regions. (C) Sequence comparison of the PEBP3-binding sites. The sequences of the three protected regions identified above are listed. The original SV40 core motif is also shown for comparison. Conserved nucleotides are boxed. Symbols on the right indicate the relative strength of binding: +++, strong; +, weak; -, inactive. The minimum consensus sequence matching with the two strong binding sites is shown at the bottom.

TABLE 1. Specificity of sequence recognition by PEBP3

Row	DNA site or fragment ^a	Sequence ^b	Binding efficiency ^c	Source or reference
		1234567		
1	Py A, W	ctGACCGCAG	+++	42, 44
2	Py B	aaAACCACAC	+++	This work
3	Synthetic	tcAACCGCAT	+++	This work
4	SV40 72/71	gcAACCATAG	+	This work
5	Synthetic	ctGACCGCGC	+	This work
6	Synthetic	ctGACCGCQC	—	This work
7	Synthetic	ctGACAGCQC	—	This work
8	SV40 core	ctTTCCACAC	—	This work
9	Py A, A2	ctGAGCACAG	—	42
10	Py A, M2	ctCACGCGAG	—	44
11	Py A, M2A	ctGAGCQCAG	—	This work
12	Synthetic	tcAAACGAAAT	—	This work
13	Synthetic	ctGCCCGTAA	—	This work
14	Synthetic	ctGCCCGTGC	—	This work
Consensus		AACCAACA		

^a All sequences listed, except those in rows 4, 8, and 9, were chemically synthesized with some flanking sequences (total length, 15' to 30 bp), cloned into pUC13 at the *Bam*HI site, and excised with *Hind*III and *Eco*RI. The resultant fragments were terminally labeled with ³²P and used for the gel retardation assay essentially as described in Materials and Methods. The fragment in row 9 with a mutated PEA2 site was tested by gel retardation assay as a 22-bp duplex (data quoted from reference 44). The sequences in rows 4 and 8, as located within the 270-bp whole SV40 enhancer fragment, were assayed by DNase I footprinting (data taken from Fig. 7B).

^b Ten-nucleotide segments of the respective sequences were aligned for maximum matching with the PEA2 site (row 1). The central 7 nt representing the recognition sequence for PEBP3 were numbered and are indicated by large capital letters. The flanking 3 nt are indicated by small capital letters. Nucleotides changed from the original PEA2 sequence are underlined and indicated by different styles of letters depending on their putative functional effects: lightface, unharmed; boldface, moderately defective; italic, severely defective.

^c Symbols indicate the relative strength of binding: +++, strong; +, weak (about one order less than that of PEA2); —, virtually inactive.

site, was detected at the junction between the 21-bp GC boxes and the 72-bp repeats (nt 101 to 113, numbered according to the method of Buchman et al. [6]). Within this region is noted a sequence motif, AACCATA, which is very similar to the one found above in the Py enhancer B element, except that the sixth nucleotide is changed from C to T. From comparison of these active and inactive binding sites, we deduced the minimal consensus sequence for PEBP3 to be PuACCPuCA (Fig. 7C). This consensus accords with the results of methylation interference and binding competition studies, which showed that mutational or chemical modifications of the first 4 bp and the sixth one are deleterious to the binding activity of PEBP3 (Fig. 3B and 4; 44). To further examine the validity of the proposed consensus, we prepared several synthetic DNA probes with variously altered consensus sequences and tested their PEBP3-binding capacities by the gel retardation assay. The results are compiled in Table 1 together with those obtained in the preceding experiments. Cross inspections of the data from 14 different sequences listed allowed us to draw a few major conclusions: (i) the 7-bp consensus must truly represent the core recognition domain, because it is conserved among the most effective binding sites, whereas adjacent nucleotides are quite variable (rows 1 through 3); (ii) positions 1 and 5 can be either G or A, which feature is conserved in all the active sites (rows 1 through 5); (iii) the remaining part of the consensus (-ACC-CA) appears to be stringently required for optimal binding because its change at one or more positions

(row 4 and below) is severely deleterious, except that the base substitution of transition type at position 6 or 7 permits reduced but significant binding (rows 4 and 5).

Mutational analysis of the function of the two PEBP2/PEBP3-binding sites. To assess the functional significance of the two PEBP2/PEBP3-binding sites detected above, we used the in vivo replication assay (36). Plasmid vector pPyOICAT designed for this assay contained the Py DNA replication origin core without the native enhancer (Fig. 8A). When the Py enhancer or its subdomain is reinserted into pPyOICAT, this plasmid becomes able to replicate in COP5 (49) or other cell lines expressing the Py large T antigen. Previous work (36) has demonstrated that the replication activation detected by this assay is closely correlated with, and hence provides a sensitive measure of, the capacity of an inserted sequence for transcriptional enhancement. Furthermore, the replication activity due to the A element was found to decrease by up to severalfold with a mutation in its PEBP2-binding site (M2 as seen in Fig. 3B and Table 1) (36). To extend this line of analysis, we introduced pairs of base substitutions designated MA and MB into the respective PEBP3-binding sites of the A and B elements on the whole Py enhancer unit and examined their effects on the enhancer-dependent Py DNA replication. These mutations, tested separately or together, caused moderate but significant decreases in the replication activity to 60 to 80% of the wild-type level (Fig. 8C). That such recognizable effects could be elicited by mutations in individual PEBP2/PEBP3 sites among multiple sequence motifs of the Py enhancer is taken to implicate substantial functional contributions of these sites to the overall enhancer activity. Notable in this connection is the fact that similar fractional inhibitions of enhancer function were also reported to occur with mutations in other positively acting sequence motifs within the Py enhancer (31, 48) or the SV40 enhancer (58). Since mutations MA, MB, and MAB showed nearly the same level of effects, the two PEBP2/PEBP3-binding sites appear to be functionally equivalent and their actions in vivo could be interdependent or cooperative to some extent, even though purified PEBP3 binds to these sites in vitro in an independent manner, as mentioned above.

DISCUSSION

We have isolated a nuclear factor PEBP3 from Ha-ras-transformed NIH 3T3 cells. This factor was originally identified (44), and currently purified, by its ability to specifically bind to the sequence motif, PEA2, in the Py enhancer A element. However, the present study showed that it can also bind to an additional site within the B element, another Py enhancer domain as important as the A element. The discovery allowed a better definition of the minimum consensus sequence for PEBP3 as PuACCPuCA. Judging from this sequence specificity, PEBP3 appears to be different from other known nuclear factors. This and other intriguing properties of PEBP3 as elucidated here broaden the scope for molecular and functional dissection of this protein factor.

The analysis with purified PEBP3 has revealed that this factor exists mainly as heterodimers of polypeptides α and β , in either the presence or absence of DNA. Analogous heterodimer formation has been reported to occur between the transcription activator protein Jun-AP-1 family and the product of oncogene *c-fos* (16, 38). Dimerization of AP-1 and Fos is mediated by the heptad leucine repeat sequence, which is commonly found in these and other dimeric transcription factors (27). More recently, a still different type of

FIG. 8. Effect of mutations introduced into each of the two PEBP2/PEBP3-binding sites on enhancer-dependent Py DNA replication. (A) Structure of pPyOICAT. Plasmid pPyOICAT contains the Py DNA fragment (nt 5267 to 152) and the CAT coding sequence. The Py fragment harbors the replication origin core and the early promoter but lacks the enhancer region. (B) Diagrams showing positions of mutations in the Py enhancer fragments tested. Fragment BPP indicates the original Py enhancer (nt 5201 to 5265). Underneath are shown the positions of base substitutions, MA and MB, introduced into the respective PEBP2/PEBP3 motifs within the A and B elements along with the original sequences. The bottom three lines indicate Py enhancer fragments containing either or both

heterodimer formation was found to occur among a novel group of DNA-binding factors, such as NF- κ B2 and MyoD1, which are characterized by their conserved helix-loop-helix structure (37). It would be interesting to see whether the α and β polypeptides of PEBP3 similarly contain the leucine repeat or the helix-loop-helix motif or represent an entirely new class on their own.

Apart from the dimerization capacity noted above, PEBP3 contrasts with other transcription factors in its mode of interaction with DNA. Most other dimeric factors characterized to date, such as AP1 (4, 30), CREB (57), GREB (25), and TREB (13), were shown to recognize palindromic sequence motifs, each subunit corresponding to a half site. Such cooperative protein-DNA interactions at two half sites is considered to enforce the specificity and efficiency of site recognition. On the other hand, evidence suggests that individual subunits of PEBP3 recognize the whole binding site as monomer: (i) the consensus sequence, PuACCPuCA, is quite asymmetric; (ii) subunit α , which was presumed to behave as a monomer, elicited exactly the same pattern of methylation interference as did the putative $\alpha\beta$ dimer. Thus, the dimeric nature of PEBP3 must have some functional significance other than that implicated above. One attractive possibility is that PEBP3 acts as a divalent DNA-binding factor. If so, PEBP3 could mediate looping or bridging of DNA when more than one cognate site are available on single or separate DNA strings, possibly providing one mechanism to allow regulatory interactions between spatially separated sequence elements (43). However, these speculations entirely remain to be tested.

Worth commenting on here is the possible relationship of PEBP3 to its counterpart, PEBP2, as detected in untransformed mouse fibroblasts (44). In consideration of the slower mobility of PEBP2 in the gel retardation assay, this factor seems to have a larger molecular size than does PEBP3 (44; Fig. 1B). Interestingly, an apparent conversion of PEBP2 to PEBP3 was found to occur when the nuclear extract from NIH 3T3 cells was treated with acid phosphatase *in vitro* or when it was prepared from the same cells but pretreated with protein kinase inhibitors *in vivo* (45). Two-dimensional gel retardation analysis (45) has further demonstrated that the PEBP2-DNA complex, once separated in the first run and treated *in situ* with acid phosphatase, gave rise to faster-moving bands corresponding to PEBP3-DNA complexes in the second run. Thus, we infer that PEBP2 and PEBP3 share a common DNA-binding core and that the former contains an extra subunit(s) whose association depends on protein phosphorylation. To examine this possibility, we are currently undertaking purification of PEBP2 by DNA affinity chromatography. Preliminary results have shown that

of the two mutations as indicated. These fragments were inserted into the BglII site of pPyOICAT with the early side (the PvuII terminus) oriented to the origin core. (C) Replication activities of pPyOICAT derivatives. COP5 cells (2×10^6 in a 100-mm dish) were transfected with 0.5 μ g of test plasmid as indicated above each lane together with 100 ng of control plasmid (pUC13). The leftmost lane (—) represents the backbone plasmid with no insert, pPyOICAT. After 24 h of transfection, replicated plasmid DNA was extracted and assayed as described in Materials and Methods. Upper bands represent replicated test plasmids, and lower bands are due to the control plasmid included to monitor the transfection efficiency. The number below each lane indicates the relative replication activity of test plasmid as determined by densitometric scanning after correction for transfection efficiency.

PEBP2 indeed contains DNA-binding polypeptides resembling the a and b subunits of PEBP3.

What regulatory function does PEBP3 or its counterpart PEBP2 confer through its binding to the Py enhancer B element in addition to the A element? The present mutational analysis using the *in vivo* replication assay indicated that both the newly found PEBP2/PEBP3-binding site and the original one positively contribute to the activation of viral DNA replication by the whole Py enhancer. This notion is consistent with and further extends the results of our previous mutational analysis centered at the A element (36). Within the A element, two other motifs binding PEBP1 and PEBP5 were determined to be mainly responsible for enhancing replication as well as transcription. As outlined in the introduction, PEBP2 is presumed to functionally cooperate with these positively acting factors, either through their mutual molecular interactions over their neighboring recognition sequences or through the putative competition of PEBP2 and another negatively acting factor, PEBP4 (12), for binding to overlapping sites. An apparent analogy may be raised for functional interrelations between PEBP2/PEBP3 and other factors binding to the B element. Previous binding studies with crude nuclear extracts from mouse NIH 3T6 cells detected a factor, designated PEB1, that binds to the central part of this region spanning nt 5180 to 5190 and is implicated at least in the activation of Py DNA replication dependent on the B enhancer element (41). Since the PEB1-binding site adjoins the second site for PEBP3 (nt 5189 to 5195), these factors could cooperate or compete with each other in controlling the enhancer activity of the B element. Thus, the recurrent involvement of PEBP2/PEBP3 in the two major enhancer elements A and B strongly suggests that these factors have pivotal roles in properly maintaining the Py enhancer activity. Further mutational analysis focusing on the dual PEBP2/PEBP3 sites should help define their exact regulatory contributions to the viral propagation.

Thus far, nothing is known about the role of PEBP2/PEBP3 in control of cellular gene expression. However, computer scanning of DNA data bases detects potential binding sites for these factors in regulatory regions of numerous eucaryotic genes. For example, there are three such sequences within the enhancer of immunoglobulin heavy-chain genes (AAACCACA/T, boxed sequences B, E, and F in Fig. 5B of Banerji et al. [3]). Another notable case is the rat growth hormone gene, which contains a PEA2-like sequence within its 5'-flanking region in juxtaposition with a half site of thyroxine-responsive element (13; GATCA GGGACGT*Q*A*C*C*G*C*A*G*G: underlines, half sites of TRE; asterisks, PEA2 homology). It would be interesting to examine whether and how PEBP2/PEBP3 affects expression of these and other genes with cognate motifs.

ACKNOWLEDGMENTS

This work was supported partly by grants-in-aid for cancer research from the Ministry of Education, Science and Culture, Japan (subject no. 63015035 for K.S. and no. 63010036 for Y.I.). This work was also partly aided by Research Grant 87-1901 of the Princess Takamatsu Cancer Research Fund for Y.I.

LITERATURE CITED

- Angel, P., E. A. Allegretto, S. T. Okino, K. Hattori, W. J. Boyle, T. Hunter, and M. Karin. 1988. Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP1. *Nature* (London) 332:166-171.
- Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* 49:729-739.
- Banerji, J., L. Olson, and W. Schaffner. 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. *Cell* 33:729-740.
- Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian. 1987. Human proto-oncogene *c-jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* 238:1386-1392.
- Borrelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature* (London) 312:608-612.
- Buchman, A. R., B. L. Burnett, and P. Berg. 1980. The SV40 nucleotide sequence, p. 799-829. In J. Tooze (ed.), *DNA tumor viruses, molecular biology of tumor viruses*, 2nd ed., part 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Chiu, R., M. Imagawa, R. J. Imbra, J. R. Bockoven, and M. Karin. 1987. Multiple *cis*- and *trans*-acting elements mediate the transcriptional response to phorbol esters. *Nature* (London) 329:648-651.
- Chodosh, L. A., R. W. Carthew, and P. A. Sharp. 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol. Cell. Biol.* 6:4723-4733.
- Davidson, I., J. H. Xiao, R. Rosales, A. Staub, and P. Chambon. 1988. The HeLa cell protein TEF-1 binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. *Cell* 54:931-942.
- de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen. 1984. Polyoma virus DNA replication requires an enhancer. *Nature* (London) 312:242-246.
- Fujimura, F. K., P. L. Deininger, T. Friedman, and E. Linney. 1981. Mutation near the polyoma virus DNA replication origin permits productive infection of F9 embryonal carcinoma cells. *Cell* 23:809-814.
- Furukawa, K., Y. Yamaguchi, E. Ogawa, K. Shigesada, M. Satake, and Y. Ito. 1990. A ubiquitous repressor interacting with an F9 cell specific silencer and its functional suppression by differentiation specific positive factors. *Cell Growth Differ.* 1:135-147.
- Glass, C. K., J. M. Holloway, O. V. Devary, and M. G. Rosenfeld. 1988. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell* 54:313-323.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
- Hager, D. A., and R. R. Burgess. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Anal. Biochem.* 109:76-86.
- Halazonetis, T. D., K. Georgopoulos, M. E. Greenberg, and P. Leder. 1988. c-Jun dimerized with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* 55:917-924.
- Hearing, P., and T. Shenk. 1983. The adenovirus type 5EA transcriptional control region contains a duplicated enhancer element. *Cell* 33:695-703.
- Hen, R., E. Borrelli, C. Fromental, P. Sassone-Corsi, and P. Chambon. 1986. A mutated polyoma virus enhancer which is active in undifferentiated embryonal carcinoma cells is not repressed by adenovirus-2 E1A products. *Nature* (London) 321:249-251.
- Herbomel, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyomavirus. *Cell* 39:653-662.
- Hirai, S.-I., R.-P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv. 1989. Characterization of *junD*: a new member of the *jun* proto-oncogene family. *EMBO J.* 8:1433-1439.
- Imler, J. L., C. Schatz, C. Wasylyk, B. Chatton, and B. Wasylyk. 1988. A Harvey-ras responsive transcription element is also

- responsive to a tumor-promoter and to serum. *Nature (London)* 332:275-278.
22. Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight. 1987. Identification of a rat liver nuclear protein that binds to the enhanced core element of three animal viruses. *Genes Dev.* 1:133-146.
 23. Kadonaga, J. T., and R. Tjian. 1986. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA* 83:5889-5893.
 24. Katinka, M., M. Yaniv, M. Vasseur, and D. Blangy. 1980. Expression of polyoma early functions in mouse embryonal carcinoma cells depends on sequence rearrangements in the beginning of the late region. *Cell* 20:393-399.
 25. Kumar, V., and P. Chambon. 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* 55:145-156.
 26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
 27. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240:1759-1764.
 28. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* 243:1681-1688.
 29. Lee, C., A. Levin, and D. Branton. 1987. Copper staining: a five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* 166:308-312.
 30. Lee, W., P. Mitchell, and R. Tjian. 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 4:741-752.
 31. Martin, M. E., J. Piette, M. Yaniv, W. J. Tang, and W. R. Folk. 1988. Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homolog and two contiguous proteins. *Proc. Natl. Acad. Sci. USA* 85:5839-5843.
 32. Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
 33. Mueller, C. R., A.-M. Mes-Masson, M. Bouvier, and J. A. Hassell. 1984. Location of sequences in polyomavirus DNA that are required for early gene expression in vivo and in vitro. *Mol. Cell. Biol.* 4:2594-2609.
 34. Mueller, C. R., W. J. Mueller, and J. A. Hassell. 1988. The polyomavirus enhancer comprises multiple functional elements. *J. Virol.* 62:1667-1678.
 35. Mueller, W. J., D. Dufort, and J. A. Hassell. 1988. Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. *Mol. Cell. Biol.* 8:5000-5015.
 36. Murakami, Y., M. Asano, M. Satake, and Y. Ito. 1990. A tumor promoting phorbol ester, TPA, enhances polyomavirus DNA replication by activating the function of the viral enhancer. *Oncogene* 5:5-13.
 37. Murre, C., P. S. McCaw, H. Vassin, M. Caudy, L. Y. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lasser, H. Weintraub, and D. Baltimore. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* 58:537-544.
 38. Nakabeppu, Y., K. Ryder, and D. Nathans. 1988. DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell* 55:907-915.
 39. Nakamaye, K. L., and F. Eckstein. 1986. Inhibition of restriction application to oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* 14:9679-9698.
 40. Perbal, B. 1988. A practical guide to molecular cloning, 2nd ed. John Wiley & Sons, Inc., New York.
 41. Piette, J., and M. Yaniv. 1986. Molecular analysis of the interaction between an enhancer binding factor and its DNA target. *Nucleic Acids Res.* 14:9595-9611.
 42. Piette, J., and M. Yaniv. 1987. Two different factors bind to the domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. *EMBO J.* 6:1331-1337.
 43. Ptashne, M. 1988. How eukaryotic transcriptional activators work. *Nature (London)* 335:683-689.
 44. Satake, M., T. Ibaraki, and Y. Ito. 1988. Modulation of polyomavirus enhancer binding proteins by Ha-ras oncogene. *Oncogene* 3:69-78.
 45. Satake, M., T. Ibaraki, Y. Yamaguchi, and Y. Ito. 1989. Loss of responsiveness of an AP1-related factor, PEBP1, to 12-O-tetradecanoylphorbol-13-acetate after transformation of NIH 3T3 cells by the Ha-ras oncogene. *J. Virol.* 63:3669-3677.
 46. Sekikawa, K., and A. J. Levine. 1981. Isolation and characterization of polyoma host range mutants that replicate in nullipotent embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* 78:1100-1104.
 47. Tanaka, K., K. Chowdhury, K. S. S. Chang, M. Israel, and Y. Ito. 1982. Isolation and characterization of polyomavirus mutants which grow in murine embryonal carcinoma and trophoblast cells. *EMBO J.* 1:1521-1527.
 48. Tang, W. J., S. L. Berger, S. J. Triezenberg, and W. R. Folk. 1987. Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. *Mol. Cell. Biol.* 7:1681-1690.
 49. Tyndall, C., G. La Mantia, C. M. Thacker, J. Favaloro, and R. Kamen. 1981. A region of the polyomavirus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. *Nucleic Acids Res.* 9:6231-6250.
 50. Velich, A., and E. Ziff. 1985. Adenovirus E1A proteins repress transcription from the SV40 early promoter. *Cell* 40:705-716.
 51. Veldman, G. M., S. Lupton, and R. Kamen. 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. *Mol. Cell. Biol.* 5:649-658.
 52. Wasylyk, B., J. L. Imler, B. Chatton, C. Schatz, and C. Wasylyk. 1988. Negative and positive factors determine the activity of the polyoma virus enhancer domain in undifferentiated and differentiated cell types. *Proc. Natl. Acad. Sci. USA* 85:7952-7956.
 53. Wasylyk, C., P. Flores, A. Gutman, and B. Wasylyk. 1989. PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes. *EMBO J.* 8:3371-3378.
 54. Wasylyk, C., J. L. Imler, J. Perez-Mutul, and B. Wasylyk. 1987. The Ha-ras oncogene and tumor promoter activate the polyomavirus enhancer. *Cell* 48:525-534.
 55. Weiher, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. *Science* 219:626-631.
 56. Yamaguchi, Y., M. Satake, and Y. Ito. 1989. Two overlapping sequence motifs within the polyomavirus enhancer are independently the targets of stimulation by both the tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the Ha-ras oncogene. *J. Virol.* 63:1040-1048.
 57. Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs III, and M. R. Montminy. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature (London)* 334:494-498.
 58. Zenke, M., T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon. 1986. Multiple sequence motifs are involved in SV40 enhancer function. *EMBO J.* 5:387-397.