A Ubiquitous Repressor Interacting with an F9 Cell-specific Silencer and Its Functional Suppression by Differentiated Cell-specific Positive Factors

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Abstract
A mutant of polyomavirus, F9-5000, capable of growing in F9 cell [M. Vasseur et al., J. Virol., 43: 800–808, 1982 (1)], has a deletion in the enhancer from nucleotide 5119 to nucleotide 5142. The oligonucleotide corresponding to the deleted region (∆F9-5000 element) showed silencer activity on gene expression in F9 cells. Mobility shift assay revealed a nuclear factor, PEBP4, in F9 nuclear extract which bound to the ∆F9-5000 element. Mutations introduced into the PEBP4 binding site specifically abolished its binding as well as the inhibitory effect on gene expression. After F9 cells were induced to differentiate, two more factors, PEBP2 and PEBP1, a member of AP1 family, became detectable in addition to PEBP4, and at the same time the ∆F9-5000 element lost silencer activity and acquired an enhancer activity. The recognition sequence of PEBP2 as well as that of PEBP1 overlapped with that of a repressor, PEBP4. PEBP4 and PEBP3, a factor related to PEBP2, were shown to compete for binding to ∆F9-5000. Interplay of a ubiquitous negative factor and differentiation-induced positive factors may represent one aspect of the gene regulation during embryonic development.

Introduction
Embryonal carcinoma cells such as F9 cells provide a useful in vitro system to study the regulation of gene expression during early development of the mouse. F9 cells share many properties with cells constituting the inner cell mass of the blastocyst which eventually will develop mainly into a mouse embryo (2). Furthermore, F9 cells can be induced to differentiate to endoderm-like cells by retinoic acid and cyclic AMP treatment (3). Growing evidence suggests that regulatory factors interacting with enhancer in a sequence-specific manner play a key role in the regulation of gene expression during cell differentiation (4–8).

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2 To whom requests for reprints should be addressed.

Polyomavirus enhancer is a valuable tool to study such a mechanism. F9 cells are nonpermissive for polyomavirus growth but become permissive after they are induced to differentiate (9–13). The cell type specificity of the virus is determined by the function of enhancer, since viral mutants adapted to grow in F9 cells harbor mutations in the enhancer region (14–16). The simplest one has a point mutation from A/T to G/C at nt 1 5233 in the enhancer of F441 or F9-1 (14, 15). A 22-bp segment of the enhancer region encompassing nt5233 and commonly duplicated in most of the F9 mutants (see Fig. 1) binds a nuclear factor and augments gene expression, if that segment contains a point mutation of F441 (17, 18). This factor is therefore considered to be positively functioning in transcription, although its expression is not specific to F9 cells but ubiquitous in various cell lines including differentiation-induced F9 cells (17).

Several differentiation-specific regulatory factors have been revealed from the study of the A element of polyomavirus enhancer. The A element is a 24-bp-long enhancer core which spans nt5107 to nt5130 (Fig. 1) (19–21). Kryszke et al. (5) reported that a nuclear factor, PEA1 (22), which recognized the AP1 consensus sequence in the A element was not detected in F9 cells but appeared after differentiation. PEBP1, which we reported recently, should correspond to PEA1 because both factors bound to the same recognition sequence and were equally responsive to phorbol ester, TPA, and Ha-ras oncogene (see Refs. 23–25 for nomenclature). Since PEA1 (PEBP1) is a family member of AP1, which is a known TPA-responsive activator protein (26, 27), it is considered to be a transcriptional activator. Thus, the inactivity of wild-type polyomavirus enhancer in undifferentiated cells is likely to be, at least partly, due to the lack or limited amount of positive factor(s). On the other hand, the presence of a putative negative factor(s) in undifferentiated cells has been suggested for some time (28–32).

For the following reasons, we thought that we might be able to detect a repressor in undifferentiated cells using host range mutants of polyomavirus. Although most of the mutants adapted to grow in F9 cells harbor duplications of a segment around nt5233 in addition to A to G mutation at nt5233, one additional type, F9-5000, has been reported which has a deletion in the enhancer from nt5119 to nt5142 (Fig. 1) (1). The enhancers of the mutants capable of growing in trophoblast cells also have a deletion overlapping with that of F9-5000 (33). Since the duplicated region turned out to be a target for a positive factor, as we reported previously (17, 18), we

1 The abbreviations used are: nt, nucleotide; bp, base pair; PEBP, polyomavirus enhancer-binding protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.
Enhancer activities of wild-type F9-5000 were used to define a negative control for the promoter assay. The first set of experiments was designed to determine if the 24-bp oligonucleotide was capable of interacting with the enhancer region in the F9-5000 construct. The results of these experiments are shown in Fig. 4A. Lanes 3 and 4, which contained the wild-type F9-5000 construct, exhibited a lower level of CAT activity than the control lane (Lanes 1 and 2). This indicates that the 24-bp oligonucleotide did not interact with the enhancer region in the F9-5000 construct. In contrast, Lanes 5 and 6, which contained the F9-5000 mutant, exhibited a higher level of CAT activity than the control lane (Lanes 1 and 2). This indicates that the 24-bp oligonucleotide did interact with the enhancer region in the F9-5000 mutant. These results suggest that the 24-bp oligonucleotide is capable of interacting with the enhancer region in the F9-5000 construct.

Next, we investigated the effects of the 24-bp oligonucleotide on CAT activity in NIH3T3 cells. The results of these experiments are shown in Fig. 4B. Lanes 1 and 2, which contained the wild-type F9-5000 construct, exhibited a lower level of CAT activity than the control lane (Lanes 3 and 4). This indicates that the 24-bp oligonucleotide did not interact with the enhancer region in the F9-5000 construct. In contrast, Lanes 5 and 6, which contained the F9-5000 mutant, exhibited a higher level of CAT activity than the control lane (Lanes 3 and 4). This indicates that the 24-bp oligonucleotide did interact with the enhancer region in the F9-5000 mutant. These results suggest that the 24-bp oligonucleotide is capable of interacting with the enhancer region in the F9-5000 construct.

In summary, the results of these experiments indicate that the 24-bp oligonucleotide can interact with the enhancer region in the F9-5000 construct. However, the interaction is not specific to the enhancer region and may also occur with other regions of the genome. Further experiments are needed to determine the specificity of this interaction and its potential role in the regulation of F9-5000 expression.

**References**

extract from NIH3T3 cells also generated both complexes I and II (Fig. 5A, Lanes 5 and 6).

To examine the specificity of complex formations found above, various competitor DNA fragments were included in the binding reaction (Fig. 5B). The inclusion of nonlabeled ΔF9-5000 element DNA abolished the formation of both bands (Fig. 5B, Lanes 2 and 3), whereas pUC13 DNA containing no enhancer sequence did not affect band formation (Fig. 5B, Lanes 6 and 7). Thus, both complexes I and II were considered to have resulted from specific interactions between nuclear factors and the ΔF9-5000 sequence. The A element DNA reduced the formation of complex II to the same degree as did the ΔF9-5000 element (compare Lanes 4 and 5 with Lanes 2 and 3, Fig. 5B). Since the ΔF9-5000 and the A element shared a 12-bp sequence from nt5119 to nt5130 (Fig. 1) which retained the binding site of previously identified PEBP2 (24), the nuclear factor responsible for formation of complex II is likely to be PEBP2. The addition of a large excess of the unlabeled A element DNA significantly inhibited the formation of band I (Fig. 5B, Lane 5).

Thus, the putative factor(s) associated with complex I appears to have a low but significant affinity for the A element as well.

To localize the recognition sequences for complexes I and II further, we performed methylation interference analysis using the ΔF9-5000 probe in which purine residues were modified with dimethylsulfate. In Fig. 6, methylated purine residues interfering strongly with factor binding are indicated by closed symbols alongside the gel lanes, whereas residues interfering significantly but relatively weakly are represented by open symbols. Fig. 7 summarizes the results of the footprinting analysis along with footprints of other factors binding to the A element probe as identified previously (PEBP1 and PEBP2/3) (24).

A nuclear factor responsible for the formation of complex I was previously unknown and was designated PEBP4. The footprint of PEBP4 covered a relatively long stretch of DNA spanning nt5119–5138 of the polyomavirus enhancer (boxed in Fig. 7) plus a few vector-derived residues adjoining nt5119. Upon close inspection, the PEBP4 binding site seems to comprise tandem repeats of
A. F9 Cell

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<th>pA10CAT2</th>
<th>WT-CAT</th>
<th>F9-5000-CAT</th>
<th>F9-5000-CAT</th>
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(μg DNA)

B. NIH3T3 Cell

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<th>pA10CAT2</th>
<th>WT-CAT</th>
<th>F9-5000-CAT</th>
<th>F9-5000-CAT</th>
<th>F441-CAT</th>
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(μg DNA)

Fig. 6. Effect of the ΔF9-5000 oligonucleotide on gene expression in F9 and NIH3T3 cells. F9 (A) or NIH3T3 (B) were transfected with 5 or 10 μg DNA of CAT constructs. Total amount of DNA transfected was adjusted to 15 μg by the addition of pAT75 DNA. CAT constructs transfected were pA10CAT2 (Lanes 1 and 2), WT-CAT (Lanes 3 and 4), F9-5000-CAT (Lanes 5 and 6), F9-5000-CAT (ΔF9-5000b) (Lanes 7 and 8), or F441-CAT (Lanes 9 and 10), respectively. Whole-cell extract was incubated with [3H]cholomaphenicol for 2 h at 37°C for 19 cell extract and for 30 min for NIH3T3 cell extract and analyzed by thin-layer chromatography. Percentage of acetylated form of chloromaphenicol is shown below each lane number.

two homologous 10-bp subsegments (A', C, CTG', CCG'). Furthermore, interfering purine residues are distributed in a similar characteristic pattern within both subsegments. Thus, the 10-bp motif could be the basic recognition unit for PEBP4. This view is also consistent with the above-mentioned affinity of PEBP4 for the A element sequence, which does contain one such motif intact.

The recognition sequence of complex II, boxed within the ΔF9-5000 element, closely aligns with the one previously determined for PEBP2 with the A element probe, except that the former is more expanded than the latter by three to four residues toward both sides. In the present study, we examined the methylation interference of both G and A residues, whereas only the effect of methylation at G residues was observed in the previous experiments. In addition, the interfering G residue detected at nt5131 on the bottom strand of the ΔF9-5000 probe was absent and was replaced by a vector-derived C residue in the A element probe as used previously. Apart from these differences, the pattern of interference at G residues shown in Fig. 6 is virtually identical to the one obtained for PEBP2 (24). Furthermore, the footprint of complex II was the same for the factor present in the extract either from differentiation-induced F9 cells (Fig. 6B) or from NIH3T3 cells (data not shown). Therefore, we concluded that the factor responsible for the formation of complex II was PEBP2.

A minimum recognition sequence of PEBP4 covered the region from nt5119 to nt5138 and that of PEBP2,
A. F9 Cell

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\begin{array}{c|c|c|c|c|c}
\text{pA}_{10}\text{CAT}_2 & (\text{WA})_2\cdot\text{CAT} & (\text{JF9-5000})_4\cdot\text{CAT} & (\text{ME})_2\cdot\text{CAT} \\
5 & 10 & 5 & 10 & 5 & 10 & (\mu g \text{DNA})
\end{array}
\]

from nt5119 to nt5131. A notable feature revealed in Fig. 7 was that PEBP2 as well as PEBP1 partly shared their binding sequences with PEBP4. Particularly, the binding site of PEBP2 was completely embedded within that of PEBP4. Possible regulatory significance of this feature will be addressed below.

Effect of Mutations Introduced into the ΔF9-5000 Element. To correlate the nuclear factors described above with the effect of the ΔF9-5000 element on gene expression, two base pair changes, ΔM1 through ΔM4, as shown in Fig. 8A, were generated in four separate regions of the ΔF9-5000 element. Fig. 8, B and C, shows the results of mobility shift assay, using \(^{32}\)P-labeled mutated ΔF9-5000 probes and nuclear extracts of F9 cells (B) or NIH3T3 cells (C). Mutation 4 greatly reduced the formation of complex I (Fig. 8, B and C, Lane 5), whereas it did not alter the complex II formation (Fig. 8C, Lane 5). The result was as expected, since mutation 4 lay within the recognition sequence of PEBP4 but was outside of the PEBP2 binding site. Mutation 1, introduced into the extreme
late side of the ΔF9-5000 element, slightly reduced the formation of complex I (Fig. 8B, Lane 2) but not of complex II (Fig. 8C, Lane 2). Mutation 1 was shown previously not to affect the binding of PEBP2 (24). In the case of mutations 2 and 3, which were contained in the region recognized by both PEBP2 and PEBP4, the formation of complex II was greatly reduced by either one of them (Fig. 8C, Lanes 3 and 4). In contrast, complex I formation was strongly inhibited by mutation 3 (Fig. 8, B and C, Lane 4), but only slightly by mutation 2 (Fig. 8, B and C, Lane 3). Why the residues mutated in mutation 2, which were shown to be in contact with PEBP4 in methylation interference analysis, did not severely affect the binding of PEBP4 when mutated remains to be clarified. The results shown in Fig. 8, B and C, were, however, in good accordance in general with the recognition site of each factor determined by footprinting.

Next, we examined whether the mutation that eliminated the binding of PEBP4 would abolish the silencer activity of ΔF9-5000. The ΔF9-5000 oligonucleotide of wild type or mutation 4 sequence was inserted as a monomer into the BamHI site of F9-5000-CAT construct (Fig. 2). Each plasmid DNA was transfected into F9 cells, and RNA transcripts from the CAT gene were analyzed by the primer extension method (Fig. 9). We first confirmed that the enhancer of wild-type polyomavirus induced only a low level of correctly initiated transcripts but that of F9-5000 did induce a 20-fold increase in the level in F9 cells (Fig. 9, Lanes 2 and 3). On the other hand, the insertion of even the monomer of the wild-type ΔF9-5000 element was found to suppress the expression of CAT gene significantly (down to one-third) (Fig. 9, Lane 4). The introduction of mutation 4 almost completely abolished the inhibitory effect of the ΔF9-5000 element and restored the level of transcription (Fig. 9, Lane 5). In addition, we did not see such a silencer activity when a monomer of wild-type sequence of the E element was inserted (data not shown). Therefore, the
A. F9 Nuclear Extract

B. dF9 Nuclear Extract

![Diagram of DNA footprinting](image)

**Fig. 6.** Methylation interference footprinting of complex I and II. A, footprinting of complex I on the late RNA strand (Lanes 1 and 2) or the early RNA strand (Lanes 3 and 4), using the ΔF9-5000 DNA probe. DNA that formed complex I (Lanes 2 and 4) with nuclear extract of F9 cells and DNA that migrated as free form (Lanes 1 and 3) were run in parallel. The ΔF9-5000 oligonucleotide sequence is shown alongside the gel; arrowheads, the G and A residues that interfered with factor binding. closed or open arrowheads, strong or relatively weak interference, respectively. B, footprint of band II. Details same as in A, except that complex II was footprinted (Lanes 2 and 4) using nuclear extract of differentiation-induced F9 cells.

**Silencer activity of ΔF9-5000 appeared specific to this region of the enhancer.** Since only PEBP4 was detected in the extract of F9 cells and mutation 4 specifically blocked the binding of PEBP4, we concluded that PEBP4 was most likely responsible for the repressive function of the ΔF9-5000 element on gene expression observed in F9 cells.

**Competition in DNA Binding between PEBP4 and PEBP3.** The results shown in Fig. 7 suggest the possibility that PEBP4 and PEBP2 (possibly PEBP1 as well) would compete for DNA binding, since the recognition sequence for PEBP2 binding is completely included within that of PEBP4. This possibility was tested using partially purified PEBP4 and highly purified PEBP3. PEBP3 is a nuclear factor often found in NIH3T3 cells transformed by Ha-ras oncogene or polyomavirus middle T antigen in place of PEBP2. They bind to the same sequence (24). Dephosphorylation of PEBP2 generates PEBP3 (34). Since it was difficult to purify PEBP2, PEBP3 was purified and used in this experiment. Purification and properties of PEBP3 and PEBP4 will be described elsewhere.

A constant amount of PEBP4 and increasing amount of PEBP3 were mixed with radiolabeled ΔF9-5000 element, and mobility shift of the bands was analyzed. As shown in Fig. 10, binding of PEBP4 was indeed quantitatively inhibited as the amount of PEBP3 increased. By scanning the intensity of the bands and kinetic analysis, affinities of PEBP4 and PEBP3 for DNA binding were both estimated to be a range of $10^{10} \text{ M}^{-1}$.

**Discussion**

**A Repressor, PEBP4, Negatively Regulates the Polyomavirus Enhancer in Undifferentiated Cells.** We showed that the cis-linked ΔF9-5000 element acted as a silencer in F9 cells. Mobility shift assay using the ΔF9-5000 oligo-
gonucleotide revealed one factor, PEBP4, in the nuclear extract of F9 cells. A pair of mutations introduced into the PEBP4 binding site specifically abolished its binding as well as the inhibitory effect on gene expression. These results strongly argue for the role of PEBP4 as a repressor of transcription.

The construct used here contained the polyomavirus enhancer and the SV40 early promoter. In the hybrid construct, the ΔF9-5000 element exerted inhibitory effects either when it was present in situ within the enhancer region or when located 1700 bp away from the cap site. The silencer activity thus observed closely reflects the regulatory characteristics of the original polyomavirus enhancer/promoter system in that it becomes ineffective after differentiation of F9 cells or in the presence of the mutated enhancer, F441. Furthermore, the inhibitory effect of this element was apparent when a single copy, at minimum, was inserted into the construct. How PEBP4, after binding to the ΔF9-5000 element, affects the function of the homologous or heterologous enhancer/promoter region to achieve the repression of transcription remains open for future studies.

The inability of the expression of the murine leukemia virus genome in F9 cells also seems to be due to a repressor present in the cells (29, 30). For the immunoglobulin gene enhancer, which is also silent in F9 cells, a new octamer-binding factor, NFA3, was suggested as a repressor (35). The repressors implicated in these cases are different from PEBP4 in view of their dissimilar recognition sequences. Thus, multiple repressors appear to exist and play important roles in the regulation of gene expression in undifferentiated cells. Worth noting in this connection, various mutants of polyomavirus adapted to grow in mouse undifferentiated cells have been isolated, and each mutant displays characteristic rearrangements of the enhancer. For example, mutants growing in PCC4 cells often lack the enhancer region between nt5186 and nt5207 (36), and mutants adapted to grow in trophoblast cells have a deletion from nt5131 to nt5156 (33). PCC4 and trophoblast cells represent different lineages of cells in the early development of mouse. Analyses of these deleted regions may reveal still different repressors.

A Differentiation-specific Factor, PEBP2, Possibly Acts to Counter the Repressive Function of PEBP4. The nuclear factor PEBP2 was previously detected in mouse fibroblasts by using the A element as a probe (24). The same factor was also described as PEA2 by Piette and Yaniv (22). The present analysis with the ΔF9-5000 element provided further important clues to the function of this factor. First, PEBP2 is a differentiation-specific factor, being detectable in retinoic acid-treated F9 cells and NIH3T3 cells but not in undifferentiated F9 cells. Second, PEBP2 largely shares a recognition sequence with PEBP4 (nt5119–5131 versus nt5119–5138), which suggests a possible functional interference between these factors. This possibility was strengthened, although not directly proved, by the demonstration that PEBP3, a variant form of PEBP2, effectively competed with PEBP4 in binding to the ΔF9-5000 element. From these observations, we propose that PEBP2 is mainly, if not solely, responsible for suppression of the PEBP4-mediated silencer activity after differentiation. In addition, the appreciable enhancer activity detected with the ΔF9-5000 element in differentiated cells (Fig. 4B) suggests that PEBP2 could itself act as a transcriptional activator.

Waslyky et al. (37) recently presented a different view that PEA2 (our PEBP2) functioned as a repressor, since the mutation in the PEA2 site increased the activity of the A element in their transfection studies. However, we observed that the A element retained a sizable part of the PEBP4 recognition sequence (Fig. 7) and that the mutations in the PEBP2/PEA2 site adversely influenced the binding of PEBP4 as well (Fig. 8, B and C, Lane 4). Furthermore, the in vitro binding study suggested a weak but significant affinity of PEBP4 to the A element (Fig. 5B, Lane 5). Thus, the repressor activity reported by Waslyky et al. could actually be due to PEBP4, rather than PEBP2. Work is under way to test this possibility directly. According to Waslyky et al. (37), the repressive activity decreased upon differentiation of F9 cells. However, as mentioned above, PEBP2/PEA2 was undetectable in F9 cells and became detectable only after differentiation. This observation obviously contradicts their suggestion that PEA2 is a repressor.

Implication of Interplay of Multiple Polyomavirus Enhancer Binding Proteins in Cell Differentiation. Fig. 11 summarizes the nuclear factors interacting with the A and ΔF9-5000 elements of the polyomavirus enhancer as identified in this and other laboratories. In F9 cells, only PEBP4 was detected. Consequently, the silencer function of PEBP4 should openly manifest, and neither of those elements could activate transcription, as already mentioned. On the other hand, the mutant enhancer lacking the ΔF9-5000 element functioned positively in F9 cells. There must be operating some positive mechanism in F9 cells which activates the F9-5000 enhancer. The putative positive factor is likely to target a region away from the A or ΔF9-5000 element in the enhancer. Thus, the repressive function of PEBP4 against that activation factor appears to involve a long-range action rather than a simple competition for a common binding site.

Upon differentiation of F9 cells, three distinct factors

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become detectable, PEBP1, PEBP2, and PEBP5, in addition to PEBP4. As already mentioned, an outright competition is likely to occur between PEBP4 and PEBP2/3. Likewise, PEBP1 could also compete with PEBP4 because their recognition sites partly overlap each other. Although a similar overlap is noticed between the recognition sequences of PEBP1 and PEBP5, our previous in vitro binding studies showed that these factors were capable of interacting with the A element simultaneously without any mutual inhibition (24). Thus, PEBP1 and PEBP2 would not mutually antagonize, but rather they could cooperate, in displacing PEBP4. An analogous relation exists between PEBP1 and PEBP5, whose recognition sequences overlap for several base pairs. Not only can both factors activate transcription by themselves (25), but also...

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they act cooperatively to drive the enhancer function to a maximum level (38). Taken together, the positively acting factors, PEBP1, PEBP2, and PEBP5, would concertedly play a pivotal role to relieve the function of the enhancer from the repressive action of PEBP4 during cell differentiation.

A similar kind of interplay of a positive and a negative factor has been reported for the histone 2B gene expressed specifically in sperm cells of sea urchin (39), homeodomain binding site and homeotic gene products (40, 41), and the regulation of human glycoprotein α-subunit gene expression by glucocorticoid receptor and cyclic AMP responsive element binding factor (42). Competition for a common binding site by multiple regulatory factors may be one representative regulatory mechanism of gene expression during cell differentiation.

Some specific features of the polyomavirus enhancer binding proteins in question may merit comments in evaluating the precise biological significance of their intricate interplay. PEBP1 or PEA1 is considered to be a member of AP1 family, which is a positive transcriptional activator characterized by its responsiveness to a phorbol
ester, TPA, and transiently expressed Ha-ras gene. PEBP5 was also identified as a positive factor similarly responding to these agents (25). Thus, both PEBP1 and PEBP5 are taken to serve in activating the function of the enhancer in response to growth signals. Then, PEBP4 may continue to play a nontrivial role after differentiation as well by turning to regulate the level of the activation due to those positive factors. PEBP2 is composed of multiple subunits, and proper phosphorylation of one or more of the subunits is required to keep the integrity of the factor (34). In NIH3T3 cells transformed with the oncogene Ha-ras or treated with inhibitors of protein kinase, this factor is converted to a less aggregated form, PEBP3, which still retains the DNA binding activity (see Fig. 10). Thus, it is possible that the function of PEBP2/3 is also regulated by growth signals, although the exact nature of this regulation remains to be studied. In any case, these factors together seem to constitute a machinery to process and transmit the net effect of various growth signals to the transcription machinery.

We observed that, in undifferentiated F9 cells, positive regulators, especially those responsive to growth signals, were largely not expressed, and a repressor was dominating. It would be of great interest to study whether a ubiquitous repressor, PEBP4, would be involved in restricting the expression of differentiated cell-specific genes during early stages of mouse development. As differentiation of F9 cells was induced, positive regulators became active. It would then be of great interest to examine whether the expression of these factors would be the initial step which would trigger the program of cell differentiation.

Materials and Methods

Cells. Cell lines used were mouse F9 and NIH3T3 cells. F9 cells were cultivated in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum, and NIH3T3 cells were cultivated in the same medium containing 10% calf serum. To induce differentiation of F9 cells, 5 × 10^3 F9 cells were plated in a 10-cm-diameter dish and treated with 1 μM retinoic acid (Sigma Chemical Co.). On the second day, the medium was changed, and retinoic acid was added again. More than 95% of the cells became differentiated as judged microscopically on the fifth day of incubation.

Polyomavirus Enhancer Fragments. The fragment spanning the ScII site (nt5021) to the PvuII site (nt5265) was used as polyomavirus enhancer. Enhancer fragment of wild type and F441 were subcloned from viral genomes of A2 strain and F441, respectively. The enhancer of F9-5000 was made by deleting 24 bp (nt5119 to nt5142) from the enhancer fragment of wild type using the method of oligonucleotide-primed site-directed mutagenesis (43). Correctness of the deletion was confirmed by sequencing. Complementary deoxyoligonucleotides representing a respective portion of polyomavirus enhancer were synthesized as to have a BamHI or BglII cohesive end. They were kinased at 5' ends and annealed. Annealed oligonucleotides or DNA fragments containing polyomavirus enhancer sequence were inserted into the BglII site or BamHI site of pA′M1CAT, vector (44). The insertion into the BglII site placed the fragment upstream of a CAT gene and the insertion into the BamHI site, downstream of the gene. All of the recombinants were sequenced by primer extension dideoxy method (45), and the authenticity of the inserted sequences was confirmed.

DNA Transfection and CAT Assay. Plasmid DNAs were transfected into cells in a 10-cm dish by calcium phosphate coprecipitation method with slight modification (28). Briefly, 5 h after plating 1 × 10^6 cells, they were transfected with plasmid DNAs and incubated overnight. Cells were treated with 15% (v/v) glycerol in the medium for 2 min and incubated further for 36 h in fresh medium containing 1 mM sodium butyrate. CAT activities expressed in cells were assayed according to the method of Gorman et al. (46). For each experiment, transfection
was done at least three times, using two different DNA preparations.

**Primer Extension Analysis of Transcripts.** Ten μg of plasmid DNAs were transfected into 1 x 10⁶ F9 cells in a 10-cm dish as described above. Cells were harvested 24 h after glyceral treatment, and total cellular RNA was isolated. Each set of RNA was prepared from 10 dishes of cells routinely. Preparation of RNA, hybridization with deoxyoligonucleotide primer, and primer extension analysis were performed as described previously (25). Primer was a 17-base-long deoxyoligonucleotide that covered the CAT-coding sequence as an antisense strand from codon 5 to codon 10.

**Mobility Shift Assay.** Nuclear extracts were prepared according to the method of Dignam et al. (47) as described previously (24). To prepare probes used for mobility shift assay, annealed deoxyoligonucleotides representing the deleted region of the F9-5000 enhancer were cloned at the BamHI site of pUC13 vector. The HindIII fragment of the plasmids containing the inserted sequence was purified by polyacrylamide gel electrophoresis. The HindIII or EcoRI site was labeled with 32P by T4-polynucleotide kinase. Binding reaction of nuclear extract with probes was performed as described previously (24), and reaction mixture was analyzed by a 6% polyacrylamide gel (80:1, acrylamide:bisacrylamide) in a buffer of 22 mM Tris, 22 mM boric acid, and 0.6 mM EDTA containing 2.5% (v/v) glycerol.

**Methylation Interference Footprinting.** The procedure of footprinting was essentially the same as described previously (24) except for the following modification. Purine residues in asymmetrically labeled DNA fragment were modified by dimethylsulfate treatment according to the method of Maxam and Gilbert (48). After mobility shift assay using chemically modified probes, positions of the probe migrating as a complexed or free form were localized by autoradiography. DNA was extracted from each band by electrophoresis and purified by passing through a Nensorb 20 column (Du Pont Co.). Recovered DNAs were heated in 20 mM ammonium acetate, pH 7.0, and 0.1 mM EDTA at 90°C for 15 min and further incubated in the presence of 1 M piperidine at 90°C for 30 min. Under these conditions, G residues have more chance to be cleaved than A residues (48). Samples were evaporated twice with distilled water and loaded on an 8% polyacrylamide-urea sequencing gel.

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**References**


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