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TATA-Dependent Enhancer Stimulation of Promoter Activity In Mice Is Developmentally Acquired

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Herpes simplex virus (HSV) thymidine kinase (*tk*) promoter activity depends on four transcription factor binding sites, one of which is a TATA box sequence, and the presence of either a *cis*-acting enhancer sequence or a transactivator protein. Studies presented here show that this TATA box was required for promoter activity only after cells began to differentiate and then only when promoter activity was stimulated by either an enhancer or a transactivator. When the HSV *tk* promoter was utilized by mouse embryos from the one-cell to eight-cell stage of development or by undifferentiated mouse embryonic stem cells, disruption of the HSV *tk* TATA box by site-specific mutations did not reduce promoter activity. This was true even when HSV *tk* promoter activity was stimulated strongly by either the embryo-responsive polyomavirus F101 enhancer or its natural transactivator, the HSV ICP4 gene product. However, stimulated expression was dependent on a distal Sp1 DNA binding site. Similarly, disruption of the TATA box did not reduce *tk* promoter activity in primary mouse embryonic fibroblasts or in immortalized 3T3 mouse fibroblasts; in fact, promoter activity was increased up to 2.6-fold. However, in these differentiated cells, stimulation of the HSV *tk* promoter by either the F101 enhancer or ICP4 protein required the TATA box. HSV *tk* promoter activity also was dependent on its TATA box in the mouse oocyte, a terminally differentiated cell with an endogenous transactivating activity. These results reveal that the need for a TATA box is developmentally acquired and depends on at least two parameters: the differentiated state of the cell and stimulation of the promoter by either an enhancer or a transactivator.

The synthesis of mRNA in eukaryotes is carried out by RNA polymerase II, a multisubunit enzyme that requires at least seven auxiliary proteins for the accurate initiation of a basal level of transcription (8, 14). In addition to this protein complex, accurate and efficient transcription of genes involves two DNA sequences referred to as promoters and enhancers (48). Promoters are *cis*-acting DNA sequences that function proximal to the start site and determine where transcription begins. Naturally occurring promoters generally are composed of multiple transcription factor DNA binding sites and are generally active in most cell types, although they may require *cis*-acting enhancers or or *trans*-acting proteins in order to achieve full activity in some cell types. Enhancers are *cis*-acting DNA sequences that function distal to the start site, independently of their orientation or position relative to the gene. Enhancers impart tissue specificity to transcription by stimulating weak promoters only in certain cell types. However, while promoters are required for transcription in every cell type from the fertilized egg to the adult mammal, the need for enhancers is developmentally acquired. Promoters and viral origins of replication that require enhancers to achieve full activity in differentiated cells also require an embryo-responsive enhancer for the same purpose in the zygotic nuclei of two-cell mouse embryos. However, when these promoters and origins are activated in the paternal pronucleus of one-cell mouse embryos, they do not (26–29, 50, 51).

Here we address the question of whether the individual DNA sequence elements that comprise a particular promoter are required constitutively throughout development or, like enhancers, their functions are acquired at different developmental stages. The herpes simplex virus (HSV) thymidine kinase gene (*tk*) promoter was chosen as a model system because it is a well-characterized promoter that uses cellular transcription factors exclusively and functions in a wide variety of mouse cell types, including cleavage-stage embryos and undifferentiated embryonic stem (ES) cells (15, 29). In addition, the HSV *tk* promoter responds to stimulation by enhancers and transactivators (6, 45). This promoter contains four transcription factor binding sites (Fig. 1): two for Sp1, one for CAAT-box-binding transcription factor (CTF), and one for TATA-box-binding protein (TBP). In its native habitat, the HSV *tk* promoter is stimulated by the *trans*-acting HSV immediate-early protein ICP4. However, the *tk* promoter also can be stimulated by *cis*-acting enhancers. For example, the polyomavirus F101 enhancer can stimulate promoters in cleavage-stage mouse embryos, embryonic stem cells, and most undifferentiated and differentiated mouse cells (29, 33). Therefore, we evaluated the effects of site-specific mutations in the *tk* promoter on its activity in various cell types representing different stages in mouse development and under conditions in which the promoter acted alone or in concert with an enhancer or transactivator.

The TATA box element is generally considered an indispensable component of those promoters that include it in their repertoire of DNA sequence elements. The TATA box facilitates binding of TBP, a protein universally required for formation of a basal-level transcription complex in all promoters (regardless of which RNA polymerase is used) and thereby facilitates alignment of the transcription complex on the DNA template (40). Consequently, those promoters that contain a TATA box element would be expected to require it under all conditions. In fact, previous analyses of the HSV *tk* promoter were consistent with this conclusion (12, 15, 16, 23, 24, 30–32). However, the data presented here show that this view is oversimplified.

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We found that the *tk* promoter did not require its TATA box in undifferentiated mouse fertilized eggs, cleavage-stage embryos, and ES cells, regardless of whether or not the *tk* promoter was stimulated by an enhancer or a transactivator. *tk* promoter activity was TATA dependent only in differentiated cells and then only when stimulated by an enhancer or a transactivator. Thus, we conclude that the need for a TATA box to achieve full promoter activity arises only in differentiated cells, in which it plays a specific role in mediating stimulation by enhancers or transactivators. These results further serve to identify conditions under which a TATA box is required for promoter activity and demonstrate that a TATA-independent promoter can convert into a TATA-dependent promoter during the process of animal development.

MATERIALS AND METHODS

Plasmids. Plasmids containing the firefly luciferase gene (pluc) linked to the HSV *tk* promoter (ptkluc) or the *tk* promoter coupled to the polyomavirus F101 enhancer (pF101tkluc) were described by Martínez-Salas et al. (29). Plasmids in which the *tk* promoter carries a site-specific mutation were described by Majumder et al. (27). pSVCAT expresses the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the simian virus 40 (SV40) promoter/enhancer (Promega). pMEX4 expresses HSV ICP4 driven by its natural promoter (39).

Injection of DNA into mouse embryos and oocytes. Isolation and culture of CD-1 mouse embryos and oocytes were carried out as previously described (29, 35). Growing oocytes were obtained from 13- to 14-day-old females. Fertilized eggs (one-cell embryos) were isolated from 8- to 10-week-old pregnant females 17 h after human chorionic gonadotrophin hormone (hCG) was injected and were cultured in the presence of 4 μg of aphidicolin (Boehringer Mannheim) per ml to arrest their development at the beginning of S phase. Two-cell embryos were isolated at 40 to 42 h after injection of hCG, at which time they had completed S phase. When these embryos were cultured in the presence of aphidicolin, they underwent cleavage into four-cell embryos and then arrested at the beginning of S phase. In the absence of aphidicolin, most injected two-cell embryos developed into morula by 44 h.

Plasmid DNA was prepared in 10 mM Tris-HCl (pH 7.6)–0.25 mM EDTA (35) to the concentration indicated, and ~2 pl was injected into one-cell embryos between 22 and 28 h after hCG injection and into two-cell embryos between 44 and 48 h after hCG injection. Embryos that survived injection were assayed for luciferase as previously described (29, 35). Each datum point represents the mean value of 40 to 150 oocytes or embryos, and the variation among individual embryos is expressed as ± standard error of the mean. While the range of luciferase activities among individual embryos could vary as much as 1,000-fold (35), the mean value obtained from several independent experiments was reproducible to within 13 to 25%. The greatest variation was observed with use of a promoter in the absence of an enhancer in cells that required an enhancer to achieve full promoter activity (e.g., two-cell embryos). The relative activity between different types of cells and different promoters was always reproducible, even when DNA injection was performed by different people.

Transfection of cultured mammalian cells. NIH 3T3 cells (ATCC 1658) were grown in Dulbecco's modified medium (DME) supplemented with 10% calf serum (DME-CS). ES cells and primary mouse embryonic fibroblasts (PMEF) were generated from mouse blastocysts and 13-day-old embryos as described by Abbondanzo et al. (1). ES cells were grown on lysed PMEF as a feeder layer in DME (Specialty Media) plus 15% heat-inactivated fetal bovine serum and in the presence of 1,000 U of ESGRO murine leukemia inhibitory factor (GIBCO BRL) per ml to prevent them from differentiating.

Transfection of DNA by the calcium phosphate precipitation method was carried out as described previously (42). Electroporation was carried out on ~2×10^7 cells that had been washed and resuspended in 300 μl of DME-CS containing 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2). Cells were combined with defined amounts of DNA containing the test plasmids and the internal standard plasmids plus 50 μg of sonicated salmon sperm that had been purified by extraction in phenol and precipitation with ethanol. Electroporation was carried out at 200 V and 1,180 μF with a Bethesda Research Laboratories electroporator at room temperature. Cells were allowed to recover for 10 min before being washed in DME-CS at room temperature by centrifugation at 296 × *g* and then plated into a 15-cm-diameter tissue culture dish. Cells were incubated overnight at 37°C in 5% CO_2. The medium was then changed, and the incubation was continued for a total of 48 h. Cells were washed twice with phosphate-buffered saline (PBS), scraped into 1 ml of cold PBS, and washed twice in 1 ml of cold PBS (with centrifugation at 4,000 × *g* for 2 min). The cell pellet was resuspended in 100 μl of cell extract buffer consisting of 0.1 M sodium phosphate (pH 7.8), 1 mM dithiothreitol, and 0.1% Triton X-100, frozen in dry ice-ethanol, then thawed at 37°C, and centrifuged at 16,000 × *g* for 5 min at 4°C. The supernatant was assayed for luciferase activity and either CAT or total protein (Bio-Rad protein assay system). The mean value of the luciferase activity from three or more experiments was normalized to either the CAT activity from the internal standard or total protein.

Assays of promoter activity in transfected cells. Luciferase assays were done on 50 μl of cell extract prepared under the conditions described above for mouse oocytes and embryos (27). CAT assays also were done on 50 μl of cell extract prepared in cell extract buffer. Each extract was incubated with 4 mM acetyl coenzyme A–0.05 μCi of [^{14}C]chloramphenicol (50 mCi/mmol; Amersham)–0.5 M Tris-HCl (pH 8.0) at 37°C for 1 h, extracted with 900 μl of ethyl acetate, and lyophilized. The pellet was dissolved in 25 μl of ethyl acetate and chromatographed on silica gel, and the enzyme product was detected by autoradiography (42).

RESULTS

The TATA box is not required for promoter activity. Previous studies of the *tk* promoter (19, 31) revealed that measuring RNA synthesis directly was not sensitive enough either to quantify promoter activity in small numbers of cells or to measure promoter activity in the absence of a transactivator. Therefore, to determine the dependency of *tk* promoter activity on its individual elements in the absence as well as in the presence of an enhancer or transactivator, and in preimplantation mouse embryos as well as in cultured cells, *tk* promoter activity was measured by linking it to the firefly luciferase gene. Expression of firefly luciferase can be quantified in single mouse oocytes and early embryos (35), thus allowing the relative activities of various promoter mutations to be compared in a wide variety of cell types.

Plasmids were constructed in which the firefly luciferase gene was linked to either the wild-type HSV *tk* promoter or the HSV *tk* promoter containing one of several different site-specific mutations (27) that had previously been created by McKnight and Kingsbury (31), some of which interrupted a

transcription factor binding site (Fig. 1). Each plasmid containing a mutant *tk* promoter was sequenced through its promoter region to confirm sequence accuracy. The effect of each mutation was evaluated in three different cell types representing different conditions that arise during mouse development. Mouse two- to four-cell embryos contain completely undifferentiated, totipotent cells, any one of which can produce a complete animal. Mouse 3T3 fibroblasts are differentiated but immortalized cells that are commonly used to analyze promoter activity in cell cultures. Mouse oocytes are terminally differentiated cells that contain an endogenous transactivator, as revealed by their ability to activate the adenovirus EIIa promoter in the absence of its normal transactivator, the adenovirus E1A protein (13).

To evaluate the relative activities of each promoter in mouse oocytes and early embryos, plasmid DNA was injected into one nucleus and the surviving ova were cultured in vitro until luciferase activity had reached its maximum level. Because it was not practical to assay for the test reporter gene as well as an internal standard in the extract prepared from individual oocytes or embryos, only luciferase levels were assayed in individual ova, and the results of 50 to 150 successful injections were combined to determine the average amount of luciferase expression per embryo or per oocyte. To evaluate promoter activity in cultured cells, plasmid DNA was electroporated into cells along with a fixed amount of pSVCAT to provide an internal standard. pSVCAT is a plasmid containing the bacterial CAT gene driven by the early SV40 promoter/enhancer region. Luciferase activity in each assay was normalized to a constant amount of CAT activity before the activities of the promoters were compared, and the level of wild-type *tk* promoter activity in each cell type was defined as 100%. In the absence of a promoter, luciferase expression was <1% of the wild-type promoter level.

The effects of site-specific mutations on HSV *tk* promoter activity in mouse embryos, fibroblasts, and oocytes revealed that some promoter elements such as Sp1 were constitutively required, while the need for other promoter elements such as CTF and a TATA box depended on the cell type in which the promoter was assayed (Fig. 1). Mutations within either Sp1 binding site (−56 to −46 or −105 to −95) reduced promoter activity 5- to 20-fold in embryos and fibroblasts and 18- to 32-fold in oocytes. The CTF binding site was required marginally in embryos and fibroblasts but strongly in oocytes. Mutation −84/−74, which disrupts five of seven nucleotides in the CTF binding site, reduced promoter activity ~2-fold in embryos and fibroblasts and ~50-fold in oocytes, while mutation −95/−85, which disrupts two of seven nucleotides in the CTF binding site, had less than a 2-fold effect in early embryos and fibroblasts but reduced *tk* promoter activity in oocytes ~8-fold.

The unexpected result was that a TATA box was required for *tk* promoter activity in oocytes but not in two- to four-cell embryos or fibroblasts (Fig. 1). Mutation −29/−18, which completely disrupted the TATA box, actually stimulated promoter activity from 1.5- to 2.6-fold in embryos and fibroblasts. A mutation (−21/−12) that altered only the terminal nucleotide also stimulated promoter activity but to a lesser extent (1.1- to 1.5-fold), and a mutation between the TATA box and the mRNA start site (−16/−6) had little effect on promoter activity. In contrast, the same two TATA box mutations reduced *tk* promoter activity in oocytes eightfold (−29/−18) and threefold (−21/−12), while the control mutation (−16/−6) again had no effect. Thus, a TATA$^-$ *tk* promoter was 16-fold more active in embryos and fibroblasts than in oocytes. These results suggested that the need for a TATA box to support the activity of a promoter depended on the differentiated state of the host cell, a conclusion that appeared to contradict previous studies in which the TATA box was reported to be required for HSV *tk* promoter activity in all cell types (12, 15, 16, 23, 24, 30–32).

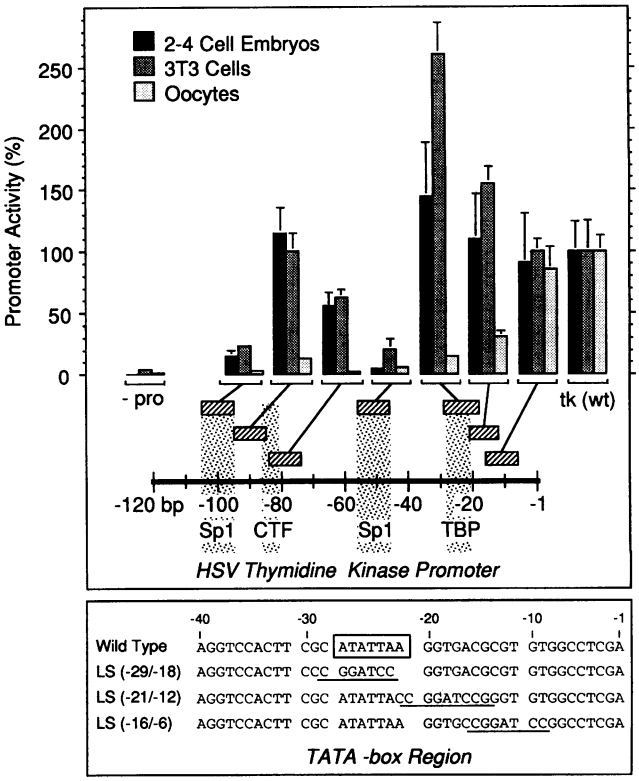

FIG. 1. Effects of site-specific mutations in the HSV *tk* promoter on promoter activity in two- to four-cell mouse embryos, 3T3 mouse fibroblasts, and mouse oocytes. Plasmid DNA (300 μg/ml) was injected into one of the zygotic nuclei of two-cell embryos, and the embryos were cultured in aphidicolin to arrest their development at the beginning of the next S phase. Plasmid DNA (2 μg) containing the mutation was combined with 2 μg of pSVCAT as an internal standard and electroporated into 3T3 cells. Plasmid DNA (500 μg/ml) was injected into the germinal vesicles of growing oocytes that were cultured in the presence of dibutyryl cyclic AMP to prevent meiotic maturation. Luciferase activities were expressed as a percentage of the amount of luciferase produced by the wild-type promoter (100%), i.e., 35,392 relative luciferase units (RLU) in two- to four-cell embryos, 3,687 RLU in 3T3 cells, and 9,073 RLU in oocytes. Error bars represent the standard error of the mean. Nucleotide positions of site-specific mutations (▨) are (right to left) −16 to −6, −21 to −12, −29 to −18, −56 to −46, −84 to −74, −95 to −85, and −105 to −95 (34), and sequence changes in the TATA box region are shown in detail (underlined). Consensus sequences for transcription factor DNA binding sites are indicated (▩). The TBP binding site is at −21 to −27, the CTF binding site is at −80 to −86, and Sp1 binding sites are at −49 to −56 and −98 to −105 (31). The luciferase gene alone (pluc) is designated −pro, and the wild-type promoter (ptkluc) is designated tk (wt).

The comparison between undifferentiated and differentiated cell types therefore was extended by comparing the amounts of luciferase produced by the wild-type *tk* promoter [ptk(wt)luc], the TATA$^-$ mutant −29/−18 [ptk(TATA$^-$)luc], and the luciferase gene alone [pluc] in S-phase-arrested mouse one-cell embryos, mouse ES cells, and PMEF (Fig. 2). Arrest of DNA synthesis by aphidicolin prevents morphological development in one-cell embryos without blocking the zygotic clock that

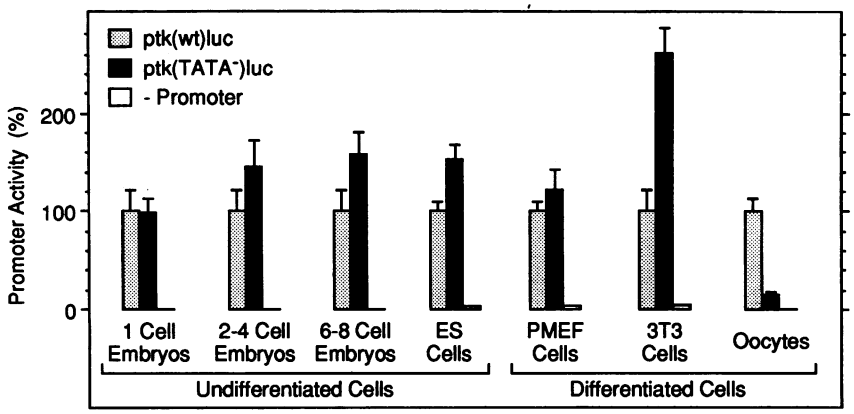

FIG. 2. Effects of disrupting the HSV *tk* promoter TATA box on promoter activity in undifferentiated and differentiated cell types. The wild-type HSV *tk* promoter [ptk(wt)], the *tk* promoter containing site-specific mutation −29/−18 [ptk(TATA⁻)], or the plasmid containing the luciferase gene alone (−Promoter) was either injected or electroporated in the indicated cells as previously described. The concentrations of plasmid DNA injected were 150 μg/ml for one-cell embryos, 250 μg/ml for two- to four-cell and six- to eight-cell embryos, and 500 μg/ml for oocytes. ES cells, PMEF, and 3T3 cells were electroporated with 2 μg of test plasmid plus 2 μg of pSVCAT. The amount of luciferase produced was expressed as a percentage of the wild-type *tk* promoter value (100%), i.e., 90,227 relative luciferase units (RLU) in one-cell embryos, 35,392 RLU in two- to four-cell embryos, 2,257 RLU in ES cells, 3,086 RLU in PMEF, 3,687 RLU in 3T3 cells, and 9,073 RLU in oocytes.

triggers the onset of zygotic gene expression at the normal time after fertilization (29, 51). Promoters injected into these arrested one-cell embryos are activated at the same time zygotic genes are expressed (29, 51). Aphidicolin-arrested one-cell embryos produce the same levels of promoter activity and require the same promoter elements that are observed in aphidicolin-arrested two- and four-cell embryos, but promoter activity in arrested one-cell embryos is not dependent on the presence of an enhancer (27). ES cells are totipotent cells derived from the inner cell mass of the blastocyst that retain their ability to colonize blastocysts and differentiate into any tissue of the animal (1). PMEF are differentiated primary cells derived from 13-day-old mouse embryos. Unlike 3T3 cells, PMEF are not immortal. Of the six cell types tested, only oocytes required a TATA box for *tk* activity; all other cells were able to utilize the *tk* promoter equally as well or even better when the TATA box element was disrupted (Fig. 2). These results suggested that *tk* promoter activity is independent of the TATA box element in most undifferentiated and differentiated cells. The special situation exhibited by oocytes is addressed later (see Discussion).

Several alternative explanations also were examined. One possibility was that the need for a TATA box depended on the ratio of promoter to cellular factors. For example, previous studies revealed that enhancer stimulation of the *tk* promoter in mouse two- to four-cell embryos depended on the amount of plasmid DNA injected: enhancer stimulation decreased 36-fold as plasmid DNA concentration was increased 10-fold, because promoter activity increased with increasing DNA concentration, while stimulation by the enhancer rapidly saturated the cell's ability to utilize the injected promoter (27). Therefore, to determine whether the need for a TATA box also depended on the concentration of plasmid DNA introduced into cells, promoter activities of ptk(TATA⁻)luc and ptk(wt)luc were compared in embryos and fibroblasts as a function of plasmid DNA concentration (Fig. 3).

As expected, promoter activity increased with increasing amounts of plasmid DNA in both cell types. However, ptk(TATA⁻)luc was consistently ~2- to 3-fold more active than ptk(wt)luc in 3T3 cells and ~2-fold more active in early embryos over a 20-fold change in DNA concentration (Fig. 3). Disruption of the TATA box never reduced promoter activity. Thus, in contrast to stimulation by an enhancer, the effect of disrupting the TATA box was relatively constant under conditions in which neither promoter ever saturated the cell's capacity to support expression of the reporter gene.

Other experimental conditions that might affect the need for a TATA box were considered. Previous studies (12, 15, 16, 23,

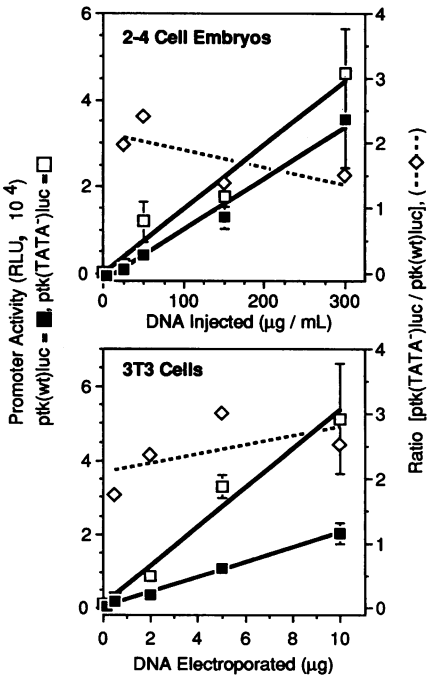

FIG. 3. Effects of plasmid DNA concentration on expression of the wild-type HSV *tk* promoter [ptk(wt)] and the *tk* promoter containing site-specific mutation −29/−18 [ptk(TATA⁻)] in two- to four-cell embryos and in 3T3 cells. Experimental conditions were as described in the legend to Fig. 1 and Materials and Methods. RLU, relative luciferase units.

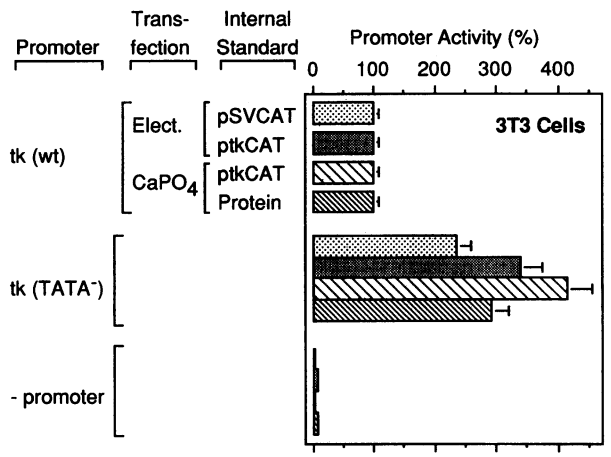

FIG. 4. Effects of the method of transfection and the choice of internal standard on luciferase production from plasmids containing only the luciferase gene [−promoter] and the luciferase gene driven by either the wild-type HSV *tk* promoter [tk (wt)] or the *tk* promoter containing site-specific mutation −29/−18 [tk (TATA$^-$)]. 3T3 cells were electroporated with 2 μg of test plasmid plus 2 μg of a pSVCAT or ptkCAT internal standard, using either electroporation (Elect.) or $CaPO_4$ precipitation ($CaPO_4$). Luciferase activities were normalized either to CAT activities or to total cell protein and expressed as a percentage of the promoter activity.

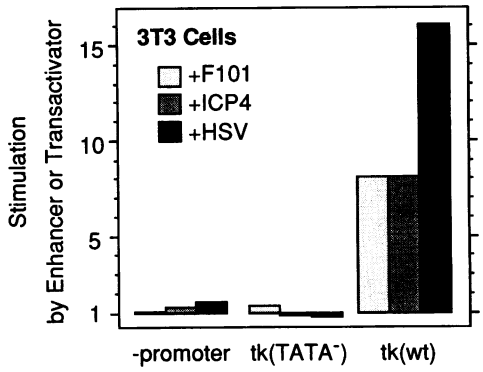

FIG. 5. Stimulation of wild-type [tk(wt)] and TATA box-disrupted [tk(TATA$^-$)] HSV *tk* promoter by HSV-infected cells (+HSV), by the HSV ICP4 gene (+ICP4), and by the polyomavirus F101 enhancer (+F101). Results with pluc (−promoter) were included as a control. 3T3 cells were electroporated (+ICP4, +F101) as described for Fig. 3 with 2 μg of test plasmid plus 2 μg of pSVCAT as an internal standard. For ICP4 stimulation, 1 μg of ICP4 expression vector pMEX4 also was included. Since electroporated cells died when infected with HSV, 3T3 cells were first transfected with plasmid DNA by the calcium phosphate precipitation method and then infected with HSV (multiplicity of infection of 1) 24 h later. Luciferase was assayed 24 h after HSV infection. Stimulation of the indicated promoter was calculated as the ratio of activity in the presence of the indicated stimulator to activity in its absence (e.g., F101tk(wt)luc/tk(wt)luc). Thus, a ratio of 1 denotes no stimulation.

24, 30–32) in human and mouse cell lines which concluded that the TATA box was required for *tk* promoter activity in all cells tested differed from those reported here in three ways. First, plasmid DNA was transfected into cells by the calcium phosphate precipitation method rather than by electroporation into cells. Since electroporation has been shown to be essential for observing bovine papillomavirus *ori*-dependent plasmid replication (46, 52), the method of transfection might affect the characteristics of promoter activity. Second, their internal standard utilized the wild-type *tk* promoter instead of the SV40 promoter. Competition for transcription factors between a mutant promoter and its wild-type counterpart might accentuate differences between their efficiencies. Third, previous studies routinely used HSV-infected cells. Since these cells contain transactivators that stimulate HSV *tk* promoter activity, a TATA box may be required only when promoters are stimulated by enhancers or transactivators.

The first two possibilities were examined in the following manner. Mouse 3T3 cells were transfected by calcium phosphate precipitation using three different internal standards, pSVCAT, ptkCAT, and total cell protein, and the results were compared with those for electroporation (Fig. 4). Regardless of the method of transfection or choice of internal standard, the ptk(TATA$^-$) promoter was always two- to fourfold more active than the ptk(wt) promoter. Similar results also were obtained when the weaker TATA mutation −21/−12 was used and when human HeLa cells instead of mouse 3T3 cells were transfected (data not shown). The third possibility is discussed below.

The TATA box is required for promoter activity when stimulated by either an enhancer or a transactivator in differentiated cells. HSV encodes an immediate-early protein, ICP4, that transactivates the HSV *tk* promoter in virus-infected cells. To determine whether *tk* promoter activity in HSV-infected cells depended on its TATA box, ptk(wt)luc and ptk(TATA$^-$)luc were electroporated into 3T3 cells, and a portion of these cells was subsequently infected with HSV. The ratio of promoter activity in HSV-infected cells to that in uninfected cells revealed that the 16-fold stimulation observed with the wild-type promoter was completely dependent on the TATA box (Fig. 5). The activity of a TATA$^-$ promoter in HSV-infected 3T3 cells was essentially the same as observed in uninfected 3T3 cells, and stimulation of luciferase gene activity was as low with a TATA$^-$ *tk* promoter as it was in the complete absence of a promoter.

To determine whether the TATA box was required for transactivation by ICP4 specifically, increasing amounts of an expression vector for ICP4 (pMEX4) were electroporated into uninfected 3T3 cells along with either ptk(wt)luc or ptk(TATA$^-$)luc. At low concentrations of pMEX4, the TATA$^-$ *tk* promoter was ~20% as active as the wild-type *tk* promoter (Fig. 6A). Moreover, while ICP4 stimulated the wild-type *tk* promoter ~8-fold, ICP4 did not stimulate the TATA$^-$ *tk* promoter at all over a 10-fold range of plasmid DNA concentration (Fig. 6B). Therefore, stimulation of the *tk* promoter by ICP4 accounted for at least half of the stimulation observed in HSV-infected cells (Fig. 5), and this stimulation was completely dependent on the *tk* promoter's TATA box.

To determine whether stimulation of the *tk* promoter by ICP4 depended only on its TATA box element, *tk* promoter mutants in each of the other three transcription factor binding sites were also examined (Fig. 7). While disruption of the TATA box eliminated stimulation by ICP4 as effectively as the absence of a promoter, disruption of the distal Sp1 binding site did not affect stimulation, and disruption of either the CTF binding site or the proximal Sp1 binding site reduced stimulation by only twofold. Therefore, in differentiated fibroblasts, stimulation by ICP4 is dependent only on the TATA box. These results are consistent with previous reports that only mutations in the TATA box significantly reduced induction of the HSV *tk* promoter, present in the HSV genome, during HSV infection (6, 23). Thus, TATA-dependent enhancer stimulation is not a function of the experimental protocol.

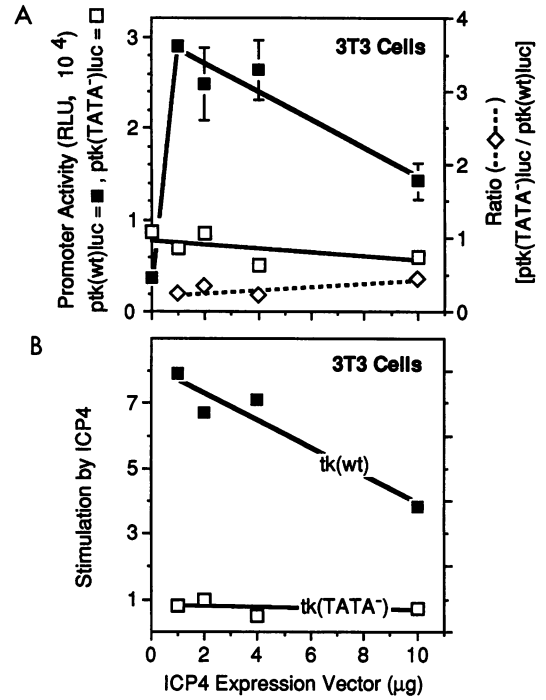

FIG. 6. Effects of ICP4 expression vector pMEX4 plasmid DNA concentration on expression of the wild-type HSV *tk* promoter [tk(wt)] and the *tk* promoter containing site-specific mutation −29/−18 [tk(TATA⁻)] in 3T3 cells. (A) Two micrograms of wild-type *tk* promoter [tk(wt)] or TATA mutant *tk* promoter [tk(TATA⁻)], 2 μg of pSVCAT as an internal standard, plus various amounts of pMEX4 were electroporated in 3T3 cells and assayed as described for Fig. 1; relative luciferase units (RLU) are plotted as promoter activity. The ratios of tk(TATA⁻) to tk(wt) for each concentration of ICP4 are also shown. (B) Stimulation of tk(wt) and tk(TATA⁻) mutant by ICP4 in 3T3 cells is calculated as the ratio of activity in the presence of ICP4 to activity in its absence. A ratio of 1 denotes no stimulation.

To determine if stimulation of the *tk* promoter by a *cis*-acting enhancer was also dependent on the TATA box, the polyomavirus F101 enhancer was positioned 600 bp upstream from the wild-type *tk* promoter [pF101tk(wt)luc] and the −29/−18 *tk* promoter mutant [pF101tk(TATA⁻)luc]. The F101 enhancer preferentially stimulates promoters such as the HSV *tk* promoter in mouse two-cell to eight-cell embryos and in mouse ES cells through its ability to bind endogenous TEF-1 (5, 29, 33). This enhancer is also active in a wide variety of other mouse cell types, including fibroblasts. In 3T3 cells, the TATA⁻ F101tk promoter was ~30% as active as the wild-type F101tk promoter over a 20-fold range of DNA concentrations (Fig. 8B). Moreover, the F101 enhancer stimulated the wild-type *tk* promoter ~8-fold but did not stimulate the TATA⁻ *tk* promoter (Fig. 8C). These results were remarkably similar to those obtained with ICP4- and HSV-infected cells (Fig. 5) and demonstrated that the TATA box was required for stimulation of the *tk* promoter in 3T3 fibroblasts by either a transactivator or an enhancer.

TATA-dependent enhancer stimulation of promoter activity is developmentally acquired. The F101 enhancer stimulated the *tk* promoter in two-cell to four-cell mouse embryos as well as in 3T3 mouse fibroblasts, but in contrast to 3T3 cells, the TATA box was not required for enhancer stimulation in cleavage-stage embryos. When pF101tk(wt)luc and pF101tk(TATA⁻)luc were injected into two-cell mouse embryos that were cultured to the four-cell stage in aphidicolin and then assayed for luciferase activity, the F101 enhancer stimulated both wild-type and TATA⁻ *tk* promoters to the same extent (Fig. 8A). Simulation of *tk* promoter activity by the F101 enhancer was independent of the TATA box throughout a 10-fold range of plasmid DNA concentrations. The same was true for ICP4 stimulation of *tk* promoter in cleavage-stage embryos (data not shown).

To determine whether stimulation of the *tk* promoter in cleavage-stage embryos was dependent on any of the *tk* promoter's transcription factor binding sites, mutants in each transcription factor DNA binding site were examined in the presence and absence of the ICP4 expression vector (Fig. 7). Inactivation of the TATA box, the proximal Sp1 DNA binding site, or the CTF DNA binding site did not reduce stimulation by ICP4. However, mutation of the distal Sp1 DNA binding site located 95 bp upstream of the transcriptional start site reduced stimulation to 5% of the wild-type *tk* promoter level. These results suggested that stimulated expression of promoters switches from an Sp1-dependent, TATA-independent mode to a TATA-dependent, Sp1-independent mode somewhere between cleavage-stage embryos and formation of differentiated cells.

To further localize the point during mouse development where enhancer stimulation became dependent on a TATA box, pF101tk(wt)luc and pF101tk(TATA⁻)luc activities were compared in two-cell mouse embryos that developed to the eight-cell stage in the absence of aphidicolin, in mouse ES cells, in PMEF, and in mouse oocytes. Except for the absence of aphidicolin, experimental conditions were the same as previously described (Fig. 1 and 2) for analysis of the *tk* promoter alone. Comparison of the luciferase activities produced by pF101tk(wt)luc with those produced by ptk(wt)luc (Fig. 9A) confirmed that the F101 enhancer stimulated *tk* promoter activity ~20-fold in two- to four-cell and six- to eight-cell embryos, ~8-fold in ES cells, PMEF, and 3T3 cells, and only ~2-fold in oocytes. Weak stimulation of the *tk* promoter by the F101 enhancer in mouse oocytes is consistent with the presence of an endogenous oocyte transactivator.

Comparison of the luciferase activities produced by pF101tk(TATA⁻)luc relative to those produced by pF101tk(wt)luc (Fig. 9B) revealed that the need for a TATA box to effect enhancer stimulation did not appear until cell differentiation was evident. Disruption of the TATA box did not affect enhancer-stimulated promoter activity in S-phase-arrested one-cell embryos, two-cell to eight-cell embryos, or ES cells. In contrast, PMEF, 3T3 cells, and oocytes showed TATA-dependent promoter activity; in each case, the TATA⁻ *tk* promoter was 15 to 25% as active as the wild-type promoter. Thus, in the presence of an enhancer, *tk* promoter activity switched from a TATA-independent to a TATA-dependent mode of operation after cells began to differentiate, probably following day 5 (formation of a blastocyst from which ES cells are derived) and before day 13 (source of PMEF).

DISCUSSION

In differentiated cells, a TATA box is required for enhancer/transactivator stimulation of promoters. Previous studies on the HSV *tk* promoter concluded that mutations in any of the four transcription factor binding sites adversely affected the ability of this promoter to function under a variety of conditions, indicating that all four sites were required constitutively for full activity (12, 15, 16, 23, 24, 30–32). Experiments reported here and elsewhere (27) using the same site-specific mutations, confirmed the need for both Sp1 DNA binding sites

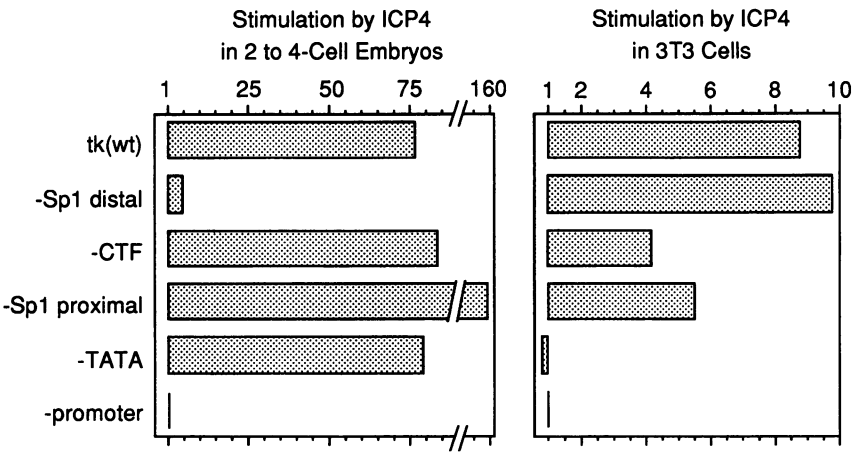

FIG. 7. Effects of site-specific mutations in the HSV *tk* promoter on ICP4-stimulated promoter activity in mouse 3T3 fibroblasts and two- to four-cell embryos. 3T3 cells were electroporated with 2 μg of test plasmid, 1 μg of ICP4 expression vector pMEX4, and 2 μg of the internal standard pSVCAT as described for Fig. 1. Two-cell embryos were microinjected with a solution containing 25 μg of the test plasmid per ml and 15 μg of either pMEX4 (expressing ICP4) or pML-1 (vector plasmid) per ml and assayed as described for Fig. 1. Transactivator stimulation was calculated as the ratio of luciferase produced in the presence of ICP4 to the amount produced in the absence of ICP4. Thus, a ratio of 1 denotes no stimulation. Mutations (Fig. 1) are −TATA (−29/−18), −Sp1 proximal (−56/−46), −CTF (−84/−74), −Sp1 distal (−105/−95), and −promoter (pluc).

and the CTF DNA binding site to achieve full promoter activity in differentiated mammalian cells such as PMEF, immortal 3T3 mouse fibroblasts, and mouse oocytes. However, in contrast to previous analyses of the HSV *tk* promoter, our results revealed that promoter activity was independent of the TATA box (Table 1). In fact, disruption of the TATA box increased the activity of the *tk* promoter alone up to 2.6-fold in 3T3 fibroblasts. The *tk* promoter was dependent on the TATA box only when the promoter was stimulated by either an enhancer or a transactivator (Table 1). In differentiated cells, disruption of the TATA box completely eliminated the ability of these transcriptional components to stimulate the promoter.

While these results at first appear to contradict earlier studies on the HSV *tk* promoter, most of the early studies were carried out under conditions in which the *tk* promoter was stimulated in order to increase mRNA synthesis to levels that are easily detectable and that allow accurate mapping of transcription start sites. For examples, most studies were carried out in HSV-infected fibroblasts or transformed cell lines (19, 31), conditions that produce HSV transactivator proteins ICP4 and ICP0, which stimulate the HSV *tk* promoter (17, 19, 41). Several studies were carried out with extracts of uninfected cells. In these studies, it was necessary to add poly(dI-dC) in order to stimulate *tk* promoter activity to measurable levels, similar to that obtained from HSV-infected cell extracts, apparently by removing some nonspecific repressor (24). Therefore, these studies reveal the requirements for a TATA box when the promoter is stimulated by a *trans*-acting factor.

Most intriguing has been the observations that the unstimulated *tk* promoter is TATA dependent in frog (15, 31) as well as mouse (Fig. 1 and 9) oocytes. These observations are consistent with the presence of an endogenous transactivation activity in oocytes, because in other differentiated cells, a TATA box was required only when the *tk* promoter was stimulated by either an enhancer or a transactivator. The weight of evidence now strongly supports the hypothesis that oocytes contain an endogenous transactivating activity. Mouse oocytes transactivated an integrated copy of the adenovirus EIIa promoter in the absence of its normally required transactivator, adenovirus E1A protein, and coinjection of the EIIa promoter and the E1A gene into mouse oocytes did not further stimulate EIIa promoter activity, presumably because the promoter is already stimulated by the endogenous transactivator (13). Similarly, coinjection of the ICP4 gene failed to stimulate the HSV *tk* promoter in mouse oocytes (data not shown), although it strongly stimulated this promoter in mouse cleavage-stage embryos (Table 1). Stimulation of other promoters by E1A has been shown to be TATA dependent in differentiated cells (43, 44), and E1A protein binds specifically and stably to TFIID (7), a protein complex that includes TBP and is required for transcription by RNA polymerase II. Thus, oocytes may contain their own version of these virus-encoded transactivators.

The presence of an endogenous generalized transactivator activity in oocytes is further supported by the observation that the SV40 early gene promoter was at least 100-fold more active in growing mouse oocytes, as judged by the production of SV40 early gene mRNA and protein, than in SV40-infected monkey cells (9), despite the fact that the SV40 early gene promoter functioned independently of its enhancer, a sequence that strongly stimulates the activity of this promoter in cultured mammalian cell lines (10). Similarly, frog oocytes produced twice the number of HSV *tk* promoter transcripts as did HSV-infected mammalian cells and 100-fold more than uninfected cells did (31). Likewise, the embryo-responsive polyomavirus F101 enhancer failed to stimulate promoters in oocytes (Fig. 9) (10, 13, 29) even though the mRNA encoding TEF-1, a critical activating protein for these enhancers (33), is present in oocytes (24a).

The question remained as to whether oocytes simply did not produce active ICP4 from the injected expression vector or contain functionally active F101 enhancer-specific activation proteins. We have recently used the strong chimeric transcription factor GAL4:VP16 to examine this question. GAL4:VP16 transcription factor activity can be quantified by its ability to stimulate a GAL4:VP16-dependent promoter, and GAL4:VP16 transactivation activity can be tested by its ability to stimulate the HSV *tk* promoter from GAL4 DNA binding sites

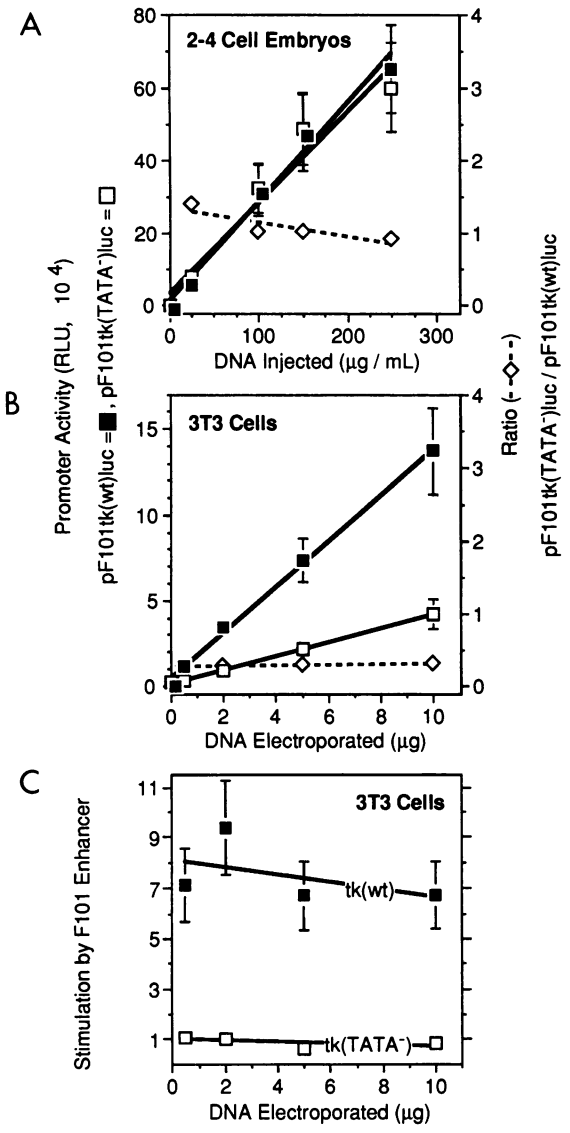

FIG. 8. Effects of plasmid DNA concentration on expression of the enhancer F101 containing wild-type [F101tk(wt)] and TATA box-mutated [F101tk(TATA⁻)] HSV *tk* promoters in two- to four-cell embryos (A) and 3T3 cells (B). The experimental conditions were as described for Fig. 1. RLU, relative luciferase units. Stimulation of the indicated promoter (C) was calculated as the ratio of activity in the presence of the F101 enhancer to activity in its absence. A ratio of 1 denotes no stimulation. Plasmids lacking a promoter produced <1% of ptk(wt)luc activity in two-cell to four-cell embryos.

placed 600 bp upstream (27). We found that saturating levels of GAL4:VP16 drove a GAL4:VP16-dependent promoter equally well in two-cell mouse embryos and oocytes, but a GAL4:VP16-dependent enhancer stimulated the *tk* promoter 30-fold in two-cell embryos and only 4-fold in oocytes (unpublished data). Therefore, the weak response to enhancers in oocytes is not due to the lack of a critical enhancer activation protein. Taken together, the results described above strongly support the presence of an endogenous transactivation activity in oocytes.

The results summarized above show that the HSV *tk* promoter requires its TATA box in differentiated cells only when it is stimulated by an enhancer or transactivator. Does this conclusion apply to all TATA-containing promoters? One example is the mouse metallothionein I gene promoter. In testis cells in which it is not stimulated by metal ion, this promoter is not dependent on its TATA box (18). Unfortunately, other studies of which we are aware characterized promoter requirements only in the presence of an enhancer. For example, the requirements for activity of the β-globin promoter, another example of a TATA-dependent promoter, were determined only when the promoter was linked to the SV40 enhancer (2, 34). However, there are many examples of TATA-dependent stimulation of promoter activity in mammalian cells. They include metal ion induction of the mouse metallothionein I gene promoter (18), Tat-dependent activation of human immunodeficiency virus gene expression (37), enhancer-dependent stimulation of the *hsp70* promoter (20), muscle-specific enhancer-dependent activation of the human myoglobin gene promoter (49), HSV-regulated expression of the HSV gC gene promoter (22), E1A-dependent stimulation of the *hsp* and c-*fos* promoters (43, 44), and AP1 enhancer-dependent stimulation of synthetic promoters (47). In addition, the *Drosophila melanogaster* actin 5C gene contains two promoters: a distal promoter that can be activated by an enhancer, and a proximal promoter that cannot be activated by the same enhancer; only the distal promoter contains a TATA box (11). High levels of *HIS4* gene transcription in *Saccharomyces cerevisiae* induced by Gcn4 is dependent on the presence of a TATA box (38). Thus, it appears that a TATA box is required only when a promoter is stimulated by either an enhancer or a transactivator, suggesting that a major role of the TATA box is to mediate the function of the enhancer or transactivator.

An alternative hypothesis would be that the TATA box is not required specifically to mediate stimulation but is required simply to achieve high levels of promoter activity. This hypothesis appears unlikely because the levels of promoter activity in mouse cleavage-stage embryos could be stimulated up to 340-fold by the F101 enhancer to levels that saturated the ability of these cells to utilize the promoter, yet disruption of the TATA box had no effect on either the stimulated or unstimulated levels of promoter activity in cleavage-stage embryos. Furthermore, the TATA box was not required in S-phase-arrested one-cell embryos in which the transcriptional capacity is equivalent to that of cleavage-stage embryos but promoters no longer can be stimulated by enhancers and therefore are apparently operating at their maximum rate (27).

Nevertheless, a TATA box alone can stimulate transcription in the absence of any other transcription factor binding sites (27). This effect most likely results from increasing the affinity of the RNA polymerase II initiation complex for the 5′ end of the gene. In addition, TATA boxes have been shown to determine the direction of transcription through the gene (25) and focus mRNA initiation events to a single site about 25 bases downstream from the TATA box (reference 4 and references therein; 36). However, in the presence of additional upstream transcription factor binding sites, the contribution of a TATA box to promoter efficiency is minor (40).

How might a TATA box mediate promoter stimulation? Although the TATA box is not a component of all promoters, TBP is a component of all basal-level transcription complexes, including those formed by RNA polymerases I and III as well as by RNA polymerase II (40). The contribution of a TATA box sequence to the composition of a promoter is to provide a DNA site with an affinity for TBP that is 10^5 times greater than that of other DNA sequences (21). Therefore, the requirement for a TATA box does not reflect a requirement for TBP but rather reflects a requirement for TBP to bind strongly and

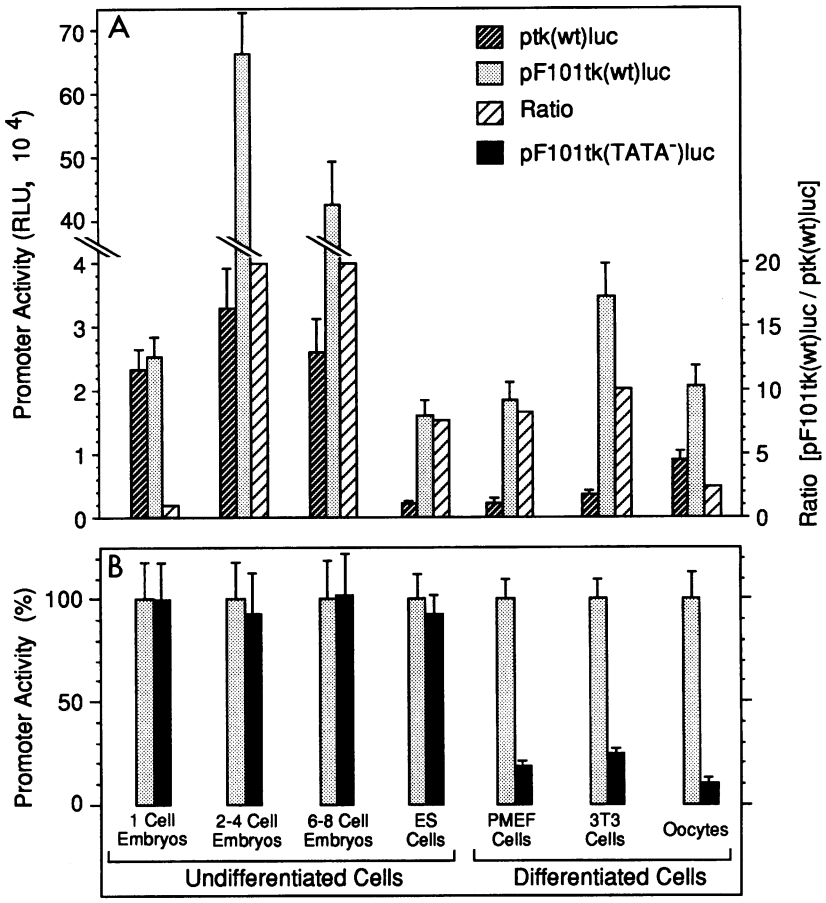

FIG. 9. TATA-dependent enhancer activation of the *tk* promoter is developmentally acquired. Experimental conditions were as described for Fig. 1. Plasmid solutions (150 μg/ml) of F101 enhancer-containing wild-type *tk* promoter [pF101tk(wt)] or TATA mutant *tk* promoter [pF101tk(TATA⁻)] expressing luciferase were expressed in one-cell embryos, and corresponding plasmid solutions (250 μg/ml) in developing two- to four-cell and six- to eight-cell embryos were expressed by microinjection. Similarly, 2 μg of each of the two plasmids was expressed in undifferentiated ES cells, differentiated PMEF, and differentiated and immortalized 3T3 cells by electroporation. Plasmid solutions (500 μg/ml) were also expressed in oocytes by microinjection. The data are represented as the relative promoter activity (A) and percent promoter activity (B). Data for ptk(wt) are also included for comparison. RLU, relative luciferase units.

specifically to a particular DNA sequence. It is this property that may be required to mediate the function of an enhancer or a transactivator. In fact, under conditions in which a TATA box was not required for ICP4 to stimulate the *tk* promoter (mouse cleavage-stage embryos), stimulation was dependent on the distal Sp1 DNA binding site (Fig. 7).

The need for a TATA box does not appear until cell differentiation occurs. Perhaps the most surprising observation was that the need for a TATA box was developmentally acquired: the need for a TATA box to stimulate the HSV *tk* promoter with either an embryo-responsive enhancer or its natural transactivator ICP4 was absent in totipotent undifferentiated cells of the early mouse embryo (Table 1). Once cell differentiation became apparent, then a switch from TATA-

TABLE 1. Conditions under which a TATA box is required to support *tk* promoter activity

Condition	Requirement for TATA box						
	Undifferentiated cells				Differentiated cells		
	1-Cell embryos	2–4-Cell embryos	6–8-Cell embryos	ES cells	PMEF	3T3 fibroblasts	Oocytes
tk promoter stimulation by:							
Polyomavirus F101 enhancer	−	+	+	+	+	+	−
HSV ICP4 gene	−	+	+	−	−	+	−
tk TATA box requirement in:							
tk promoter	−	−	−	−	−	−	+
tk promoter + enhancer	−	−	−	−	+	+	+
tk promoter + ICP4	−	−	−			+	+

independent to TATA-dependent stimulation of promoters occurred. Furthermore, stimulation of the *tk* promoter in undifferentiated early mouse embryos was mediated through the distal Sp1 DNA binding site instead of the TATA box. This finding suggests that the interactions involved in TATA-dependent stimulation of promoters in differentiated cells can be functionally replaced by Sp1 in undifferentiated cells.

Developmental acquisition of TATA box dependence for enhancer stimulation of promoters provides a mechanism by which TATA-less promoters can respond to enhancers during early development but not in differentiated cells. This could provide a way for promoters expressing housekeeping genes to be stimulated by an enhancer or a transactivator in undifferentiated cells but not in differentiated cells, thus permitting higher levels of these enzymes at the beginning of development but maintaining a low constitutive level in differentiated cells. Housekeeping genes are genes whose products are ubiquitously required in all cells, although their activities may be required at higher levels at specific times during the cell cycle or during specific stages of animal development. Most housekeeping genes are regulated by TATA-less promoters (3) that contain more than one GC box (possible Sp1 DNA binding site) and thus resemble a TATA$^-$ *tk* promoter. The fact that stimulation of HSV *tk* promoter activity relied solely on a distal Sp1 site in undifferentiated embryonic cells but on a TATA box in differentiated cells provides a paradigm for enhancer regulation of housekeeping genes.

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