Different Regulation of the Human Thymidine Kinase Promoter in Normal Human Diploid IMR-90 Fibroblasts and HeLa Cells*

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Transcriptional activation of the human thymidine kinase (hTK) promoter plays an important role in the cell cycle control of thymidine kinase expression. Using the luciferase reporter cotransfection assay, we found that the activity of the hTK promoter in IMR-90 normal human diploid fibroblasts was increased by the constitutively over-expressed cyclin A or cyclin E but not by cyclin D, suggesting that the former two cyclins may act as positive regulators for the hTK promoter. The sequence responsible for the transcriptional activation by cyclin E was identified to be located between −133 and −92 of the hTK promoter. Regulation of the hTK promoter in HeLa cells appeared to be different from that in IMR-90 fibroblasts. Firstly, the hTK promoter in HeLa was already highly activated and could not be further activated by ectopically expressed cyclin A or E. Secondly, the −133 to −92 region of the hTK promoter was important for the promoter strength in HeLa cells but not in IMR-90 cells. The steady-state levels of cyclins A and E were readily detected in HeLa cells but not in normal IMR-90 fibroblasts. Based on these results, we propose that the cellular environment of the HeLa cell allows the hTK promoter to stay fully activated for transcription regardless of ectopically expressed cyclin A or E and that transcriptional activation of thymidine kinase gene is deregulated in these tumor cells.

Thymidine kinase (TK),¹ a crucial enzyme in the salvage pathway of thymidine triphosphate formation, is indirectly involved in DNA replication. The level of TK activity is known to be increased at the G1/S phase of the cell cycle (1, 2). Several mechanisms, including transcriptional activation (3–7), post-transcriptional processing (8–10), and increase of translational efficiency (11–13), have been proposed to account for the precise timing associated with the induction of TK activity at the G1/S phase in normal cells. When the hTK promoter fused with a CAT reporter gene was transfected into Chinese hamster ovary fibroblasts by stable transfection, the sequence between −109 and −84 of the hTK promoter was found to be responsible for the TK transactivation during the G1/S transition period (14). Furthermore, several complexes containing cyclin A, p107, and p33[cdc2](15, 16) that would bind to this DNA region were detected in the nuclear extracts isolated from growth-stimulated Chinese hamster ovary fibroblasts (15). These results prompted us to investigate the relationship between the expression of G1 cyclins and the transcriptional activation of the hTK promoter in human cells.

Expression of cyclins A, D, and E has been shown to be an important driving force for the G1 progression during the cell cycle (for review see Ref. 16). Many studies demonstrated that perturbations in G1 cyclin expression caused inappropriate cell division, which would result in the development of cancer. For example, the cyclin A gene is the site of the integration of a fragment of the hepatitis B virus genome in hepatocellular carcinoma (17). Over-expression of cyclin D1 was shown to be a result of chromosomal rearrangement, translocation, retroviral insertion, and gene amplification in parathyroid tumors, lymphomas, squamous cell tumors, and breast and colorectal carcinomas (18–24). Cyclin E was recently found to be over-expressed in cultured breast cancer cell lines and in primary breast tumors, and the concentration of cyclin E increased in breast tumor cells as the disease progressed toward severity (25). Presumably, aberrant expression of these G1 cyclins could propel cells through critical transitions in the cell cycle. Also, the steady-state level of TK mRNA in normal human fibroblast has been shown to be increased in response to serum stimulation and appears to be closely associated with the stringent cell cycle control (26, 27). In contrast, in HeLa cells TK mRNA in different phases of the cell cycle is constitutively and highly expressed (13, 27). Furthermore, the level of TK activity was often found to be elevated in neoplastic tissues (28); however, it is still unclear whether or not this phenotype in tumor cells is due to deregulation at the level of transcriptional control and related to the abnormal expression of G1 cyclin. In this study, therefore, we examined the in vivo effect of over-expression of human cyclins A, D1, and E on the hTK promoter activity in normal diploid IMR-90 fibroblasts as well as in HeLa cells, a tumor cell line, and characterized the regulation of the hTK promoter in these two different cell types.

MATERIALS AND METHODS

Cell Culture—IMR-90 human embryonic lung diploid fibroblasts (passage number 5; population doubling level, 12) were obtained from the Institute for Medical Research (Camden, NJ). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone) at 37°C in 5% CO2 incubator and subcultured to obtain cultures with a population doubling level of 30–40 remaining.

Transfection and Reporter Gene Assay—Cells were incubated with a mixture of 2.1 μg of plasmid DNA and 12 μg of lipofectamine (Life Technologies, Inc.) in 1 ml of Dulbecco’s modified Eagle’s medium for 6 h. The medium was then replaced with fresh Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and incubated further for 24 h. Cells were washed three times with phosphate-buffered saline and lysed with an additional 100 μl of reporter lysis buffer (Promega). Cell lysates were centrifuged for 10 min at 10,000 × g to remove the debris. 10 μl of the supernatant were added with 100 μl of luciferase assay buffer (29). The luminescence was measured in a Packard liquid scintillation counter. Protein concentration was determined.
directly from the cell lysate using the Bradford assay.

Recombinant Plasmid Construction—The DNA fragment containing – 133 to +33 and +91 to +33 of the TK promoter sequence, respectively, was inserted in the sense orientation into the Bgl II site of a luciferase vector, pGL-2-Basic (Promega), to obtain p(–133/+33)TK-Luc and p(–91/+33)TK-Luc. Plasmids, pCMV cyclin A, pCMV cyclin D1, and pCMV cyclin E, were constructed by cloning the cDNAs encoding cyclins A (30), D1 (31), and E (31), respectively, into pCDM8 (32) at the Xho I site. To achieve this, the vector DNA and the cyclin cDNA fragments isolated from the corresponding plasmid after EcoRI digestion were all treated with Klenow enzyme to form blunt ends. They were then ligated by the conventional method. The insert orientation in the new recombinant plasmids was determined by restriction enzyme digestion. The vector DNA and the cyclin cDNA fragments were ligated into pGL-2-Basic (Promega), to obtain p(GL-2-Basic cyclin A), p(GL-2-Basic cyclin D1), and p(GL-2-Basic cyclin E), respectively.

RNase Protection Analysis—Total RNA was hybridized to a riboprobe, 46 °C overnight. The hybridized mixture was exposed to RNases A and T1 at 37 °C and analyzed in 4% polyacrylamide-urea gel. The hTK promoter was protected a 410-base pair hTK promoter region (34). The 200-base pair TK-Luc fragment purified from pGL-2-Basic SK vector (Stratagene) was linearized at the Pvu I site and added to a transcription reaction mixture containing T7 RNA polymerase and [α-32P]CTP for the synthesis of the TK riboprobe from the cDNA. This TK riboprobe protected a 410-nucleotide transcript derived from hTK mRNA in the assay. The human β-actin riboprobe was generated in a transcription reaction containing the template pTRI-β-actin-Human (Ambion) linearized at the Hind III site. This human β-actin riboprobe protected a 250-nucleotide transcript derived from β-actin mRNA in the assay.

DNase I Footprint Analysis—Nuclear extracts of cells were prepared by the method of Dignam et al. (34). The 200-base pair EcoRI– Apal fragment purified from pGL-2-Basic SK vector containing the 160–base pair TK promoter region (–133/+33) was labeled at one end by Klenow enzyme at the EcoRI site with [α-32P]dATP and used in the binding reaction as described previously (35). At the end of the binding reaction, 100 μl with 3 units of DNase I (Worthington) in 10 μg MgCl2 were added and incubated for 60 s at room temperature, and the reaction was stopped by the addition of an equal volume of the buffer containing 8 μl urea, 0.5% SDS, and 5 mM EDTA. The digested probe was extracted with phenol/chloroform and precipitated by ethanol. Samples were then analyzed in 7 μl urea-polyacrylamide sequencing gel. G+A chemical sequencing reaction of the DNA probe was performed by the method of Maxam and Gilbert (36).

Western Blot Analysis—Cells grown on a 60-mm dish were washed with phosphate-buffered saline and lysed at 4 °C by sonication in TNE buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, and 0.1 mg/ml of each leupeptin and aprotinin). The lysate was centrifuged at 10,000 × g for 15 min to remove the debris and 50 μg of protein in the lysate were separated in 10% SDS-polyacrylamide gel, followed by electrophoretic transfer onto a polyvinylidene difluoride membrane (Millipore). After a pretreatment with 5% skim milk, the membrane was incubated with the indicated antibodies. The antibodies and their dilutions were: rabbit polyclonal anti-human cyclin D1 (UBI), 1:200; monoclonal anti-human cyclin A (Santa Cruz), 1:5000; and monoclonal anti-human cyclin E (Santa Cruz), 1:3000. The alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) was used for the interaction with anti-human cyclin D1 antibody. The alkaline phosphatase color development was performed according to the procedure recommended by the manufacturer (Promega). The horseradish peroxidase-conjugated goat anti-mouse IgG was used for the detection of anti-human cyclins A and E monoclonal antibodies with an enhanced chemiluminescence method (Amersham Corp.). Exposure time was 30 s for cyclin E and 2 min for cyclin A.

RESULTS

The hTK Promoter Can Be Activated by Cyclin A or Cyclin E but Not by Cyclin D1 in IMR-90 Fibroblasts—In an attempt to test whether or not there was a direct link between the activity of the hTK promoter and expression of G1 specific cyclins in human cells, recombinant plasmids containing respective cDNA encoding human cyclin A, D1, or E, whose expression would be under the control of the cycleromegalovirus early promoter, were constructed. Each of these G1 cyclin-expressing plasmids was cotransfected into cells with a reporter plasmid, which contained a luciferase gene under the control of either the hTK promoter (–133 to +33 region, p(–133/+33)TK-Luc) or the SV40 promoter (pSV40-Luc). The effect of cyclin A, D1, or E in increasing amounts on the hTK promoter in IMR-90 fibroblasts was examined. All data were expressed relative to the luciferase activity from cells transfected with pSV40-Luc and a control expression vector, pCDM8. The results are shown in Fig. 1. Cyclin A enhanced the luciferase activity of p(–133/+33)TK-Luc in a dose-dependent manner. Up to a 3.5-fold increase was seen when 0.3 μg or more of pCMV cyclin A was used (Fig. 1A). Cotransfection with pCMV cyclin D1 up to 0.6 μg did not promote the hTK promoter activity (Fig. 1B).

A significantly increased level of cyclin D1 was detected by Western blot analysis in the IMR-90 fibroblast transfected with pCMV cyclin D1, an indication of ectopic over-expression of cyclin D (Fig. 1C). Cyclin E was also found to steadily increase the luciferase activity of p(–133/+33)TK-Luc reporter, reaching 3-fold when 0.6 μg of pCMV cyclin E was used for cotransfection (Fig. 1D). All parallel experiments performed with the pSV40-Luc construct showed some repression when increasing amounts of these three pCMV-cyclins were used for cotransfection. Thus, activation of the hTK promoter by cyclin A or E in IMR-90 fibroblasts appears to be a specific event.

Next, p(–91/+33)TK-luc, with a region from –133 to –92 deleted, was introduced by cotransfection either with pCMV cyclin A or pCMV cyclin E into IMR-90 fibroblasts to test whether or not the upstream sequence is involved in the activation. In the cells with this deletion construct, no significant increase in luciferase activity was seen with pCMV cyclin E, whereas a stimulation in response to pCMV cyclin A was still observed with this deletion construct (Fig. 2), suggesting that the region between –133 and –92 contained the element involved in the activation by cyclin E but not by cyclin A. Thus, the activation mechanism elicited by these two cyclins may be different. Furthermore, the upstream sequence did not seem to play an important role in the strength of the promoter activity in this normal cell strain, because no decrease in luciferase activity in cells transfected with p(–91/+33)TK-luc was found when compared with that in cells with p(–133/+33)TK-Luc.

Little Enhanced the hTK Promoter Activity by Cyclin A, D, or E in HeLa Cells—The in vivo effect of G1 cyclins on the hTK promoter was also examined in HeLa cells. All data were similarly expressed relative to the luciferase activity under the control of the SV40 promoter in HeLa cells. In contrast to the results observed in IMR-90 fibroblasts, none of cyclins A, D, and E significantly enhanced the hTK promoter activity in this tumor cell line (Fig. 3, A, B, and C). Clearly, the luciferase activities expressed from the hTK relative to the SV40 promoters were already high without ectopic expression of cyclins in HeLa cells. In fact, the activities of either the hTK or SV40 promoters steadily decreased as the cells were cotransfected respectively with increasing amounts of the plasmids that express these three G1 cyclins.

Correlation of the Level of the hTK Promoter Activity and the Amount of TK mRNA in HeLa Cells—The basal activity of the hTK promoter appeared to reach nearly 60% of the activity expressed from the SV40 promoter in HeLa cells, whereas it reached only 17% in IMR-90 fibroblasts, indicating that the hTK promoter was more active in HeLa cells. Table I summarizes the luciferase activities (in light counts) expressed by the SV40 promoter and the hTK promoter, respectively, in these two cell types. To verify whether or not the dramatic difference in promoter activity can be correlated with the steady-state level of TK RNA, the quantity of TK RNA in proliferating HeLa and IMR-90 cells was compared using RNase protection assay. As expected, the amount TK RNA in HeLa cells was clearly much more than that in IMR-90 fibroblasts (Fig. 4). We then transfected p(–133/+33)TK-Luc and p(–91/+33)TK-Luc, respectively, into HeLa cells to examine whether or not there was a difference in luciferase activity expressed from these two reporter plasmids. As shown in Fig. 5, a 50%
reduction of luciferase activity with the deletion construct was clearly seen, suggesting that an activation process through the −133/−92 sequence is required for the maintenance of the maximum promoter activity in HeLa cells.

Footprint Analysis of the Human TK Promoter—Because the −133 to −92 region of the hTK promoter contributed to the promoter strength in HeLa cells but not in IMR-90 cells, it is possible that the capacity of cognate factors binding to the promoter region was different between HeLa and IMR-90 cells. To determine this, the DNase I footprint analysis was carried out. When the nuclear extract prepared from serum-stimulated IMR-90 fibroblasts was used, the protected region was seen to span from −29 to −84, including the distal and proximal CCAAT boxes (Fig. 6, lanes 1 and 2), indicating that cyclin D1 was expressed in the transfected cells used in B, D, cotransfection with pCMV-cyclin E.

**Fig. 1.** Effect of cyclin A, cyclin E, or cyclin D1 on the hTK promoter in IMR-90 fibroblasts. IMR-90 fibroblasts were transfected with 1.5 μg of p(−133/+33)TK-Luc (■) and the indicated amounts of pCMV cyclin A, D1, or E, respectively, to which a complementary amount of control vector, pCDM8, was added to make a final amount of the expression plasmid mixture of 0.6 μg. Parallel transfection experiments were performed with pSV40-Luc (○). The luciferase activity was measured in duplicate and counts in cpmlmg were normalized by background counts from cells transfected with pGL-2-Basic in all cases. Individual numbers were divided by the values obtained from cells transfected with pSV40-Luc plus pCDM8 vector DNA to give percentage. Data are the averages from four experiments, and the error bars represent the standard deviation from the mean. A, cotransfection with pCMV cyclin A. B, cotransfection with pCMV cyclin D1. C, Western blot analysis of cyclin D1 for cell extract (35 μg of protein) from IMR-90 fibroblasts transfected with pCDM8 vector (lane 1) and pCMV cyclin D1 (lane 2), indicating that cyclin D1 was expressed in the transfected cells used in B, D, cotransfection with pCMV-cyclin E.

**Fig. 2.** Effect of cyclin A or E on the deleted hTK promoter. The reporter construct p(−133/+33)TK-Luc or p(−91/+33)TK-Luc (2 μg) were cotransfected with 0.3 μg of pCDM8 vector (control), pCMV cyclin A (cyclin A), or pCMV cyclin E (cyclin E), respectively, into IMR-90 fibroblasts. The luciferase activity is expressed as the percentage of that obtained from p(−133/+33)TK-Luc and pCDM8 vector. Each bar is the average of two independent experiments.
lanes 6 and 7). Apparently, the protein binding pattern with the hTK promoter was quite different in these two cell types. Unlike with the extract of HeLa cells, there seemed to be no stable proteins bound to the 5′-flanking sequences upstream from 284 of the hTK promoter in the extract of IMR-90 cells. To some extent, this may explain that this upstream region did not contribute to the promoter activity in this normal cell strain.

The Steady-state Levels of Cyclins A, D1, and E in HeLa Cells and IMR-90 Fibroblasts—Because deregulation of each G1 cyclin has been suggested to cause derangement of the cell cycle (16), here we investigated whether or not the steady-state level of cyclin A, D1, or E was correlated with the level of the hTK promoter activity in these two cell types. Lysates containing the same amount of protein prepared from semi-confluent cultures of IMR-90 fibroblasts and HeLa cells, respectively, were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blot detection with anti-cyclin A, cyclin D, or cyclin E monoclonal antibodies, respectively. The results showed that cyclin A was almost undetectable in IMR-90 fibroblasts but readily detectable in HeLa cells (Fig. 7A), whereas the level of cyclin D1 was rather similar in both cell types (Fig. 7B). As to

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**FIG. 3.** Effect of G1 cyclins on the hTK promoter activity in HeLa cells. Reporter construct p(−133/+33)TK-Luc or pSV40-Luc (1.5 μg) was cotransfected with the indicated amounts of pCMV-cyclin A, D1, or E, respectively, to which a complementary amount of control vector, pCDM8, was added to make a final amount of the expression plasmid mixture of 0.6 μg. Values are the average of four experiments. Expression of luciferase activity was as described in the legend to Fig. 1.

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**FIG. 4.** Level of TK RNA in HeLa and IMR-90 cells. RNase protection experiments were carried out with total RNA isolated, respectively, from HeLa cells and IMR-90 fibroblasts at semi-confluency. 2 μg of RNA sample were hybridized with the β-actin probe, which protected a 250-nucleotide transcript from β-actin RNA. 5 μg were used in hybridization with hTK-probe, which protected a 410-nucleotide transcript from TK RNA. The hybridized sample after RNases A and T1 digestion was electrophoresed in 4% polyacrylamide-urea gel (see “Materials and Methods”). The autoradiographic exposure times for β-actin probe and hTK probe were 4 and 24 h, respectively.

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**FIG. 5.** Comparison of luciferase activity from p(−133/+33)TK-Luc and p(−91/+33)TK-Luc in HeLa cells. HeLa cells were transfected with p(−133/+33)TK-Luc or p(−91/+33)TK-Luc (1 μg) together with equal amount of the RSV-CAT plasmid, which contained the CAT gene under the control of the Rous sarcoma virus long terminal repeat. Extracts were prepared 24 h after the transfection and assayed for luciferase activity as well as CAT activity. Luciferase activity was normalized to the CAT activity in the same sample and expressed as percentage of that from p(−133/+33)TK-Luc.

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**TABLE I**

Luciferase activities expressed by reporter constructs p(−133/+33)TK-Luc or pSV40-Luc in HeLa and IMR-90 cells

| Reporter plasmid | Luciferase activity | IMR-90 | HeLa |
|------------------|---------------------|--------|------|
| pSV40-Luc        | 4.81 × 10⁴ cpm/μg of protein | 3.19 × 10⁵ |
| p(−133/+33)TK-Luc| 8.06 × 10⁵           | 1.90 × 10⁵ |
cyclin E, there were two polypeptides detected in HeLa cells with molecular sizes of 62 and 52 kDa, of which only the former (62 kDa) was observed in IMR-90 fibroblasts (Fig. 7). When IMR-90 fibroblast transfected with pCMV cyclin E, polypeptides of sizes 52 and 42 kDa were specifically over-expressed (Fig. 7D). The detection of the 62 kDa polypeptide also appeared in cells transfected with pCDM8, indicating that it was a nonspecific signal. Thus, cyclin E (the 52-kDa band) was readily detectable in HeLa cells but not in IMR-90 fibroblasts. Taken together, it seemed to show that unlike in HeLa cells, the amount of endogenous cyclins A and E was rather limited in the proliferating IMR-90 fibroblasts.

**DISCUSSION**

The results presented here establish four points: (i) cyclin A or cyclin E may act as the positive modulator of hTK promoter activity in normal IMR-90 human diploid fibroblasts; (ii) the transcriptional activation stimulated by cyclin E is via a region between –133 and –92 of the hTK promoter; (iii) the activity of the hTK promoter is much higher in HeLa cells and cannot be further induced by ectopic expression of cyclin A or E; and (iv) the region between –133 and –92 is required for the maximum promoter activity in HeLa cells but not in IMR-90 fibroblasts. These data also suggest that regulation of the hTK promoter in tumor cells is different from that in normal human cells and that the loss of transcriptional control of the TK gene is one of the events relevant to the deregulation at the G/S transition of the cell cycle in tumor cells.

Here, cyclin D expression seemed to exert little effect on the activation of the hTK promoter in both IMR-90 and HeLa cells. Because the activity of cyclin D-cdk4 complex occurs rather early during the G1 progression (37), it is conceivable that cyclin D may not be involved in the transactivation of the hTK promoter directly. Furthermore, the steady-state level of cyclin D, unlike that of cyclin A or cyclin E, can readily be detectable in IMR-90 fibroblasts, suggesting that the expression of cyclin D is not as limited as that of cyclin A or cyclin E in this normal cell strain. In a normal cell cycle, cyclin E-ckd2 association is
required for the G1/S transition (38, 39), whereas cyclin A-cdk2 association is needed for the S phase (40, 41). Apparently, the trans-activation of the human TK promoter is well coordinated with these two temporally coupled events at the G1/S transition during the cell cycle progression. Data presented here would support the model that the transcriptional complex formation involving cdk2, cyclin A, or cyclin E is critical for the transcriptional activation of cell cycle-regulated genes at the G1/S and S phases. Luciferase activities expressed from p(−133/+33)TK-Luc and p(−91/+33)TK-Luc showed little differences in IMR-90 fibroblasts (Fig. 2), suggesting that the deleted region (−133/−92) is not involved in the negative control; it is, however, needed for positive control by cyclin E. The sequence alteration in −84 to −109 of the hTK promoter has been shown to abolish G1/S phase regulation of the reporter gene that was under the control of the hTK promoter in the stably transfected Chinese hamster ovary cells (14). This −84 to −109 region contains two potential binding sites for transcription factor E2F and two Yi-like binding motifs (42). It remains to be seen whether or not ectopically expressed cyclin E, by forming a transcriptional complex with E2F or Yi-like factors, activates the hTK promoter via this upstream sequence. The activation mechanism by cyclin A, on the other hand, seems to differ from that by cyclin E, because the deletion of the −133 to −92 region did not affect the stimulation by cyclin A.

Our results indicated that the hTK promoter can be fully activated in IMR-90 only when there is a sufficient amount of cyclin A or cyclin E by ectopic expression (Fig. 1). In contrast, in HeLa cells it seems that all factors are already present for the transactivation. In other words, the cellular environment of HeLa cells it seems that all factors are already present for the transactivation of the hTK promoter via this upstream sequence. The activation mechanism by cyclin A, on the other hand, seems to differ from that by cyclin E, because the deletion of the −133 to −92 region did not affect the stimulation by cyclin A.

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