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Transcriptional Activation of the Human C-Myc Gene by Simian Virus 40 Large T Antigen without Binding to p53 and RB Proteins in the Transient Expression System

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Transcriptional activation of the human c-myc gene by SV40 large T antigen was examined using HepG2 cells by co-transfecting a T antigen expression plasmid with a myc-CAT construct containing the 2.3-kb upstream region from the P1 promoter and the P2 promoter region fused to the CAT gene. T antigen increased the basal activity of the P2 promoter region containing the E2F binding site, but both the P2 promoter region and the upstream region from the P1 promoter were important for overall activation by T antigen. CAT assay using mutated T antigen lacking p53 or the RB binding site indicated that p53 or RB was not mainly involved in transcriptional activation of the c-myc gene. It appears that activation of the c-myc gene by T antigen is probably dependent upon E2F and a cellular factor through a mechanism which is independent of binding of T antigen to p53 and RB.

The c-myc gene belongs to an immediate early response gene and plays a major role in $G_0/G_1 \rightarrow S$ transition in the cell cycle (1). In fact, over-expression of the c-myc gene promotes disorderly proliferation of the cells. It is also known that the level of c-myc RNA is low in quiescent cells, but elevates after stimulation by growth factors, such as PDGF or FGF (2).

The c-myc gene is known to have four promoters at least (P0, P1, P2, and P3). P2 is generally the predominant promoter, giving rise to 75-90% of c-myc RNAs, whereas the others generate 10-25% of c-myc RNAs (3, 4). Numerous cis-acting elements regulating these promoters and the effects of a number of oncoproteins on the c-myc gene transcription have been reported (5). For example, the ME1a2-E2F-ME1a1 (myc exon1, a1 and a2 sites) elements located between the P1 and P2 transcription start sites of the mouse c-myc gene upregulate transcription from the P2 promoter (6, 7). Hiebert et al. (8) showed that transcription of the human c-myc gene from the P2 promoter was activated by adenovirus E1A protein, and that this activation depended on the E2F binding site (GGCGGGAAA) located between the P1 and P2 start sites. Batsche et al. (9) showed that activation of the human c-myc promoter by simian virus 40 (SV40) large T antigen in fibroblast and epithelial cell lines depended on the interaction of T antigen with RB, and that the E2F binding site was necessary for this activation. Moberg et al. (10) reported that a mutated p53 protein activated the expression of murine c-myc P2 promoter-driven reporter gene, while wild type p53 suppressed the myc-CAT construct. Based on the previous findings, it was surmised that activation of the c-myc gene transcription by T antigen is probably through a mechanism, which is dependent on binding of T antigen to p53 or RB protein.

In this study, we examined transcriptional activation of the human c-myc gene by T antigen using human hepatoblastoma HepG2 cells by co-transfecting a T antigen expression plasmid with a myc-CAT construct containing the 2.3-kb upstream region from the P1 promoter and the P2 promoter region that was fused to the coding region of the chloramphenicol acetyl transferase gene (CAT). It was found that transient expression of T antigen was able to increase the basal activity of c-myc gene transcription from the P2 promoter region and that for overall activation of the c-myc gene by T antigen, both the P2 promoter region and the 1.2-kb upstream region from the P1 promoter were necessary. Furthermore, our deletion mutant analysis using a variety of myc-CAT constructions driven by the P2 promoter and wild type or mutated T antigen gene lacking p53 or RB binding site demonstrated that activation of the c-myc gene transcription by T antigen was independent of p53 or RB.

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MATERIALS AND METHODS

Cell culture. HepG2 cells, derived from a human hepatoblastoma (11) were maintained at 37°C in the DM-160AU medium (Kyokuto) supplemented with 10% (v / v) fetal calf serum and kanamycin (60μg / ml).

Plasmids. Construction of pmycCAT, pmycPCAT, pmycCCAT, pmycP2CAT and pmycHP/P2CAT has been described previously (12, 13). pUSVT was constructed by insertion of the 3-kb KpnI-BamHI fragment of SV40 DNA which contains the SV40 enhancer, early promoter, T antigen ORF, and poly(A) addition signal, into the pUC19 vector (Takada et al., unpublished data). Mutant T antigen expression plasmids pMT-A2 and pEF321-NKLT were the gifts from Dr. S. Sugano of the Institute of Medical Science, Tokyo University, and pK1 was provided by Dr. J. A. Decaprio of Harvard Medical school.

Plasmid (pRIB750) containing the 750-bp riboprobe template for the RNase protection assay was constructed as follows. Firstly, pmycP2CAT was digested with PvuII, that yielded two fragments (1.0-kb and 4.5-kb). The 1.0-kb fragment was then digested with SmaI, and 0.7-kb SmaI-PvuII fragment was inserted into the HindII site of pBluescript SK(−) (Stratagene).

DNA transfection. DNA transfection was carried out by the calcium phosphate precipitation method (14) using 5 μg of myc-CAT DNA as the reporter plasmid and 15 μg of T antigen expression plasmid. The cells were plated at 7.5×10^6 per 10 cm dish, cultured for 24 hr before DNA transfection, then incubated with DNA-calcium phosphate precipitates for 6 hr at 37°C. Each transfection experiment was carried out in duplicate and repeated at least three times.

CAT assay. After 48 hr culture cell extracts were prepared as described (15), and subjected to CAT activity assay using [^{14}C] chloramphenicol as the substrate. At least three independent experiments were separately carried out in each case and a typical result is shown in each figure. Chloramphenicol and its acetylated derivatives were separated by ascending thin-layer chromatography on a silica-gel plate [$CHCl_3$-CH_3OH, 95 : 5 (v/v)] and visualized by autoradiography. To quantify the reaction products, radioactive spots were cut from the silica-gel plate and counted in a liquid scintillation counter.

RNase protection assay. The riboprobe was synthesized using pRIB750 and T7 RNA polymerase (16). Total RNA was prepared by Isogen kit (Nippon gene), and subjected to the RNase protection assay basically as described (16). Samples containing riboprobe and 150μg of total RNA were denatured at 85°C for 10 min, then hybridized at 45°C for 8 to 12 h. RNase treatment was carried out with 40μg/ml RNaseA (Sigma) and 1000U/ml RNaseT1 (Boehringer Mannheim) at 33°C for 90 min. The RNAs were precipitated with ethanol after phenol extraction. Protected RNA fragments were analyzed on a 4% polyacrylamide/8M urea gel.

Preparation of low-molecular-weight DNA for DpnI assay. Low-molecular-weight DNA was prepared from the transfected cells similar to the method of Hirt (17). Cell pellets were suspended in 900μl of 20 mM Tris-HCl (pH7.8)/10 mM EDTA, then 60μl of 10% SDS and 250μl of 5M NaCl were added. After incubation at 4°C overnight, the solution was centrifuged at 17,000 g for 30 min at 4°C. The aqueous phase was extracted with phenol followed by ethanol precipitation. The pellets were dissolved in 90μl of sterile water, and treated with RNaseA for 1 hr at 37°C. The DNAs were precipitated wiath ethatnol twice after phenol extraction. The DNAs were digested with DpnI and BamHI, and subjected to electrophoresis in a 1% agarose gel and Southern blot hybridization. The hybridization probe of 300-bp CAT ORF was made by PCR amplification with CAT primers, and ^{32}P-labeled by nick translation. The primer sequences were : 5′-CTA TAA CCA GAC CGT TCA GC-3′ for the sense strand and 5′-GTT TTC ACC GTA ACA CGC-3′ for the anti-sense strand.

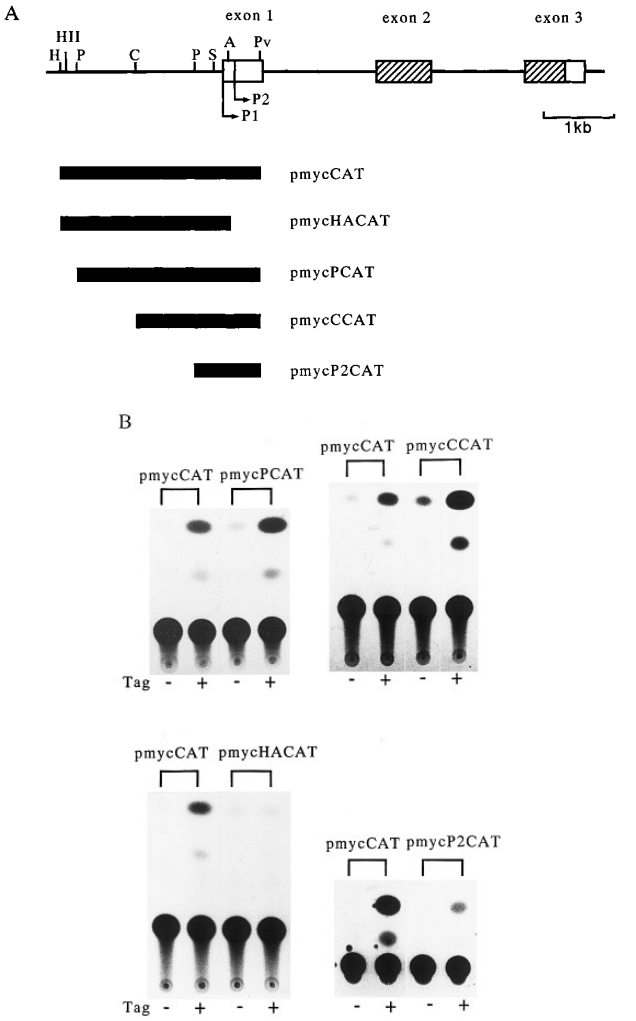

FIG. 1. (A) Structure of myc-CAT constructs. Abbreviations: H, HindIII (nt.2328); HII, HpaII (nt.2246); P, PstI (nt.2119 and nt.409); C, ClaI (nt.1256); S, SmaI (nt.93); A, ApaI (nt.40); Pv, PvuII (nt.517); P1, P1 promoter; P2, P2 promoter. The restriction map of the human c-myc gene is shown at the top. (B) CAT assay for various myc-CAT constructs. The assay was carried out as described in Materials & Methods.

RESULTS

c-Myc gene expression was activated by SV40 large T antigen dependent upon the P2 promoter region and the upstream region from the P1 promoter. To examine whether T antigen activates expression of the c-myc gene, pmycCAT containing exon1 and the 2.3-kb upstream region from the P1 promoter fused to the CAT ORF (Fig. 1A) and T antigen expression plasmid pUSVT were co-transfected into HepG2 cells. Co-transfection of pUSVT increased CAT activity 7-fold than that of the control as an average of three independent experiments (Fig. 1B). The data clearly indicated that T antigen can activate c-myc gene expression.

T antigen-response region was then searched using

several deletion mutants of pmycCAT and results are shown in Fig. 1B. CAT activity of pmycPCAT was activated to 5.2-fold than that of the control, where pUC19 was co-transfected instead of pUSVT. The level of activation was slightly decreased when compared with pmycCAT. CAT activity of pmycCCAT was activated 4.2-fold compared with the control. Overall CAT activity of pmycP2CAT was much decreased, but was still activated 3.8-fold by pUSVT co-transfection. On the other hand, pmycHACAT, that lacks a region containing the P2 start site, exhibited no significant activation by T antigen. These results suggested that activation of the basal activity was dependent on the PstI/PvuII region (nt.409∼nt.517) of the c-myc gene, while enhancing effect was present in the ClaI/PstI region (nt.1256∼nt.409), because the overall CAT activity was much decreased by deletion of this region. As CAT activity was not observed after deletion of the ApaI/PvuII region (nt.40∼nt.517) containing the P2 promoter, the P2 promoter region may be essential for the basal activity, but not sufficient for the overall activity. The 1.2-kb upstream region from the P1 promoter is required for the overall activity of c-myc gene.

The data suggested that T antigen activates transcription from the P2 promoter of the c-myc gene, and that overall activation of the c-myc gene by T antigen was likely brought about by a combination of two T antigen response regions, the P2 promoter region and the 1.2-kb upstream region. Moreover, there is no E2F binding site (sequence : GCGGGAAA) in the 1.2-kb upstream region from the P1 promoter.

T antigen activated transcription from the P2 promoter of the c-myc gene. An RNase protection experiment was carried out to determine a transcription start site from the P2 promoter. pmycCAT and pUSVT were co-transfected into HepG2 cells under the same conditions used in the CAT assay and total cellular RNA was prepared from transfected cells and subjected to RNase protection assay. As shown in Fig. 2, 496-nt transcript derived from the P2 promoter (P2-initiated CAT transcript) of transfected reporter plasmid was protected and the amount was increased by pUSVT co-transfection compared with the control (lanes 3 and 4). Besides, 356-nt transcript derived from the P2 promoter of endogenous c-myc gene (P2-initiated endogenous c-myc transcript) was also protected and its amount was increased by pUSVT (lanes 6 and 7). Data indicated that T antigen co-expression increased the basal transcriptional level from the P2 promoter of the transfected reporter plasmid and the endogenous c-myc gene as well. On the other hand, the transcript from the P1 promoter should be detected by the 750-nt riboprobe used in this experiment, however, transcripts from the P1 promoter of the transfected reporter plasmid and the endogenous c-myc gene were not actually detected. Bands between 496-nt (P2-initiated CAT transcript) and 356-nt (P2-initiated endogenous c-myc transcript) did not correspond to transcripts from the P1 or P0 promoter.

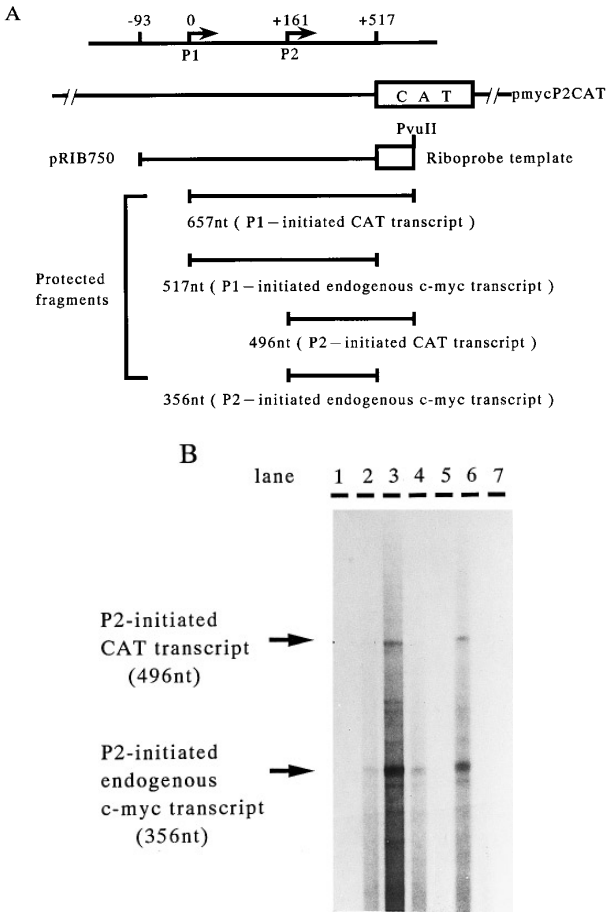

FIG. 2. Analysis of the protected RNA from transfected HepG2 cells. (A) Outline of the 750-bp probe and protected RNA fragments. The 750-nt probe extends from the −93 position of the c-myc gene to the PvuII site of CAT DNA. (B) RNase protection assay. The protected 496-nt and 356-nt bands derived from the P2 promoter in transfected plasmids and endogenous c-myc are shown by arrows, respectively. Twenty micrograms of yeast tRNA was used as the negative control (lane 1). Lanes 2 and 5 show isolated RNA from non-transfected HepG2 cells. Lanes 3 and 6 show co-transfection of pmycCAT and pUSVT. Lanes 4 and 7 show co-transfection of pmycCAT and pUC19. One hundred and fifty micrograms of total RNA in lanes 2, 3, and 4 or 50 μg of total RNA in lanes 5, 6, and 7 was used.

Replication of myc-CAT DNA was undetectable. Augmentation of CAT activity can be observed when the copy number of the CAT reporter plasmid is increased by replication of the plasmid DNA. Therefore, we investigated whether or not augmentation of CAT activity was due to increase in the copy number of the reporter plasmid. Low-molecular DNAs were prepared from transfected cells under the same conditions used in the CAT assay, and subjected to the DpnI assay. If transfected pmycCAT DNA is methylated, pmycCAT DNA

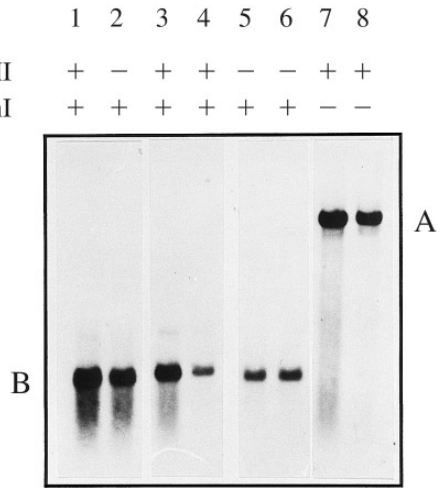

FIG. 3. DpnI assay of transfected reporter plasmid pmycCAT. pmycCAT was used as the reporter plasmid in all lanes. A indicates the BamHI-digested band of 8 kb. B indicates the DpnI-digested band. The assay was carried out as described in Materials & Methods. Lanes 1 and 2 show co-transfection of pUC19 and digestion with DpnI and BamHI. Lanes 3 and 4 show co-transfection of pUSVT and digestion with DpnI and BamHI. Lane 5 or 7 shows co-transfection of pUC19 and digestion with DpnI or BamHI only. Lanes 6 and 8, respectively, show co-transfection of pUSVT and digestion with DpnI or BamHI only.

can be cleaved by DpnI at multiple sites. On the other hand, if its copy number is increased in HepG2 cells by T antigen, replicated DNAs are resistant to DpnI digestion, hence, subsequent digestion with BamHI, that cuts one site in pmycCAT, should give a single 8.0-kb fragment. As shown in Fig. 3, all the DNAs were cleaved with DpnI, and the DpnI-resistant band of 8.0-kb was not detected. Therefore, it was concluded that the augmentation of CAT activity by T antigen is not due to replication of the myc-CAT DNA.

T antigen activation of the c-myc gene was independent of binding to tumor suppressor gene product p53 and RB. It is well known that T antigen binds to tumor suppresser gene products of p53 and RB and inhibits their functions. Since the E2F binding site between the P1 and P2 start sites of the c-myc gene was reported to be a T antigen response element (9, 18), it is interesting to see whether this is the case in our system. Mutant pMT-A2, where Arg residue is inserted between aa.425 and aa.426 of T antigen ORF, expresses T antigen that is unable to bind to p53 (19). pEF321-NKLT is a mutant that lacks aa.110∼aa.152 of T antigen ORF, where the RB binding site (aa.102∼aa.115) and a nuclear localization signal (aa.126∼aa.132) reside (20). Mutant pK1 is substituted Lys for Glu at aa 107 of T antigen ORF and expresses T antigen that is unable to bind to RB (21). Each T antigen mutant plasmid and pmycCAT reporter plasmid were co-transfected into HepG2 cells. As shown in Fig. 4, co-transfection of pMT-A2 or pK1 increased CAT activity of pmycCAT similar to pUSVT. However, co-transfection of pEF321-NKLT did not activate CAT activity of pmycCAT. Data indicated that the nuclear localization of T antigen was necessary for activation, but the binding of T antigen to p53 and RB was not involved in the transcriptional activation of c-myc gene. Similar activation experiments were carried out by co-transfecting pUSVT and myc-CAT constructs in human osteosarcoma Saos-2 cells, where both p53 and Rb genes were mutated. Again, we observed 4-fold activation (data not shown). It appears that overall activation of the c-myc gene transcription from the P2 promoter by T antigen is probably independent of binding to p53 and RB proteins. Present data suggested that the T antigen response element in the P2 promoter region corresponds to the E2F binding site. Consistent with the previous

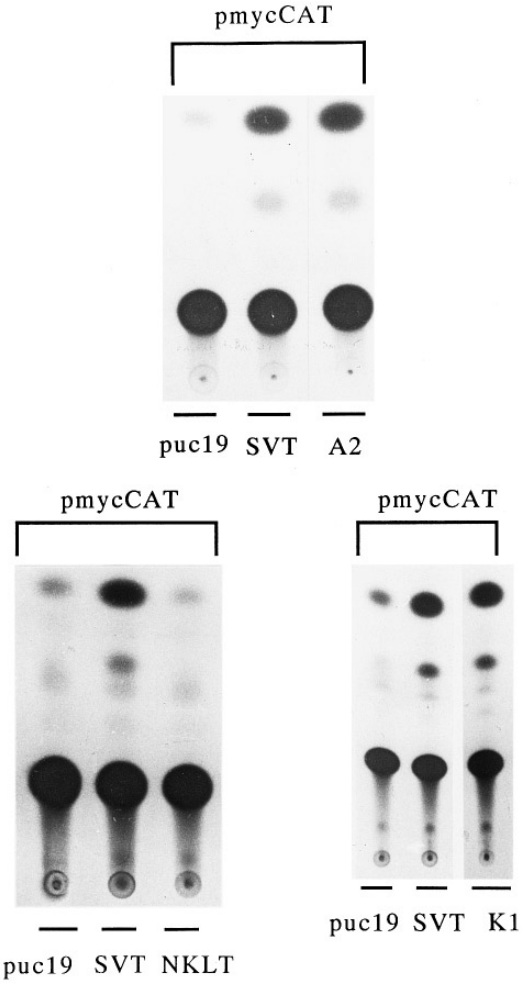

FIG. 4. CAT activity assay using the mutant T antigen expression plasmid. pmycCAT was used as reporter plasmid in all lanes. The assay was carried out as described in Materials & Methods. pMT-A2 expresses p53-binding-defective T antigen. NKLT is the mutant T antigen lacking a nuclear localization signal. pK1 expresses the mutant T antigen lacking RB-binding activity.

result (19), the nuclear localization of wild type T antigen and the cytoplasmic localization of NKLT mutant were confirmed by immnofloresent staining (data not shown).

DISCUSSION

In this study, the transient transfection experiments using HepG2 cells demonstrated that SV40 T antigen activated transcription of the c-myc gene, and T antigen-response regions were determined using a variety of mutant myc-CAT constructs. One was in the P2 promoter-containing region (nt.40∼nt.517) and the other was in the 1.2-kb region upstream of the P1 promoter (nt.1256∼nt.409). The P2 promoter region was essential for activation of the basal activity, but not sufficient for overall activation. Since the nt.40∼nt.517 region contains the P2 promoter and its transcription start site, the requirement for this region suggests that T antigen activates transcription from the P2 start site. To confirm this point, an RNase protection mapping was carried out, and it was found that T antigen activated transcription from the P2 start site.

Recently, some *cis*-acting elements which regulate transcription initiation from the P2 promoter have been reported (18). Increased level of transcription from the mouse c-myc gene P2 promoter was dependent upon the ME1a2-E2F-ME1a1 region between the P1 and P2 transcriptional start sites, and the E2F binding site was involved in the activation of the c-myc gene by T antigen. Batsche et al. (9) also obtained the same result in the human c-myc gene. Requirement for the RB binding site of T antigen was also investigated, but the results were negative. There is no element related to T antigen, such as ME1a2-E2F-ME1a1, in the 1.2-kb upstream region of the P1 promoter. Therefore, interaction of T antigen with E2F and other cellular factor may be involved in overall transcriptional activation of the c-myc gene by T antigen.

CAT assay using mutant T antigen, which was unable to bind to p53 and RB, indicated that transcriptional activation of the c-myc gene by T antigen in the present system was independent of p53 or RB binding. CAT assay using a plasmid that expresses T antigen without a nuclear localization signal indicated that nuclear localization of T antigen was essential for the transcriptional activation of the c-myc gene by T antigen. The data from the present study suggest that T antigen activates overall transcription from the P2 promoter of c-myc gene by means of interaction of T antigen with the basal transcription machinery and by interaction of T antigen with E2F and other cellular factor. Further analyses on the P2 promoter region and their binding proteins are required to understand the molecular mechanism of T antigen activation of the c-myc gene as well as that of other cellular genes.

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