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NOTES

Simian Virus 40 T Antigen Can Transcriptionally Activate and Mediate Viral DNA Replication in Cells Which Lack the Retinoblastoma Susceptibility Gene Product

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Simian virus 40 T antigen is a multifunctional protein which has recently been shown to form a complex with the retinoblastoma susceptibility gene product (Rb protein) (J. A. DeCaprio, J. W. Ludlow, J. Figge, J.-Y. Shaw, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston, Cell 54:275–283, 1988; P. Whyte, K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow, Nature (London) 334:124–129, 1988). This interaction may facilitate some of the functions of T antigen. The ability of simian virus 40 T antigen to mediate transcriptional activation and viral DNA replication was tested in human osteosarcoma cell lines U-2OS and Saos-2, which are Rb positive and Rb negative, respectively. Both functions of T antigen were efficient in both cell lines. Hence, these functions can occur in the absence of Rb protein.

The early proteins of a variety of DNA viruses form complexes with specific cellular proteins (5, 10, 14, 15, 24). These interactions are believed to be significant for the transforming functions of at least two viral proteins, adenovirus E1a and simian virus 40 (SV40) large T antigen (5, 24). Recent studies of one of these cellular proteins have identified it as the product of the retinoblastoma susceptibility gene (p105-RB, hereafter referred to as Rb protein) (5, 24). This protein appears to be involved in the control of cell proliferation, and it has been suggested that the function of Rb protein may be abrogated through its association with the viral transforming proteins (5, 24).

SV40 large T antigen is a multifunctional protein which, during lytic infections, participates in the control of viral gene transcription and viral DNA replication (1, 3, 13, 16, 18, 20). T antigen must stimulate the infected cell to grow in order to induce cellular replicative enzymes needed for the amplification of viral DNA. In addition, T antigen is a promiscuous transcriptional activator, causing the activation of the SV40 late promoter as well as many nonviral promoters (1, 3, 13). Although T antigen is a well-characterized DNA-binding protein (17, 21), it need not bind to DNA for transcription activation to occur. It appears that T antigen induces transcriptional activation by abrogating a cellular process, which results in the modification of existing cellular transcription factors or the induction of cellular transcription factors (2, 6). Therefore, T-antigen-mediated activation of both viral and cellular promoters can be explained by the broad activating effect T antigen has on cellular transcription factors.

It is possible that the transcriptional activation and viral DNA replication functions of T antigen are related to association with the Rb protein. To assess this possibility, we tested T-antigen-mediated transcriptional activation by using human osteosarcoma cell lines which are either Rb protein positive or negative. We found that transcriptional activation of several promoters occurred efficiently in each cell line. Since these are primate cell lines, plasmids which contained an intact SV40 origin of replication amplified in the presence of T antigen. We found that replication also occurred efficiently in each cell line. Hence, our data suggest that T antigen can mediate its transcriptional activation and viral DNA replication functions in the absence of Rb protein.

Human osteosarcoma cell lines U-2OS (Rb^+ [11]) and Saos-2 (Rb^- [11]) were the gifts of S. Friend. Cells were grown in Dulbecco minimal essential medium plus 10% fetal calf serum. Monolayers at 80% confluence were transfected by the calcium phosphate procedure, except that HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered phosphate was replaced with BES-buffered phosphate (25 mM BES [pH 6.95], 140 mM NaCl, 0.75 mM Na_2PO_4) (4). The following chloramphenicol acetyltransferase (CAT) transient-expression plasmids were transfected: (i) pL2-CAT, with the CAT gene controlled by the SV40 late promoter (this construction contains a wild-type SV40 origin of replication) (13); (ii) pL2n-CAT, which is the same as pL2-CAT, except that the origin of replication has been made nonfunctional because of a 6-base-pair deletion at the *BglI* site in the origin (7, 13); (iii) pKcat23, the CAT gene controlled by the adenovirus E3 promoter (23); (iv) pRSV-CAT, the CAT gene controlled by the promoter of the Rous sarcoma virus (RSV) long terminal repeat (LTR) (8); (v) pSV0-CAT, the CAT gene with no promoter (9) (this is a negative-control plasmid and is used as an inert plasmid filler for transfections). The activator plasmids were p6-1 ΔL (7, 13), which contains the SV40 early region and expresses both small t and large T antigens, and the E1a-producing plasmid pE1a (19). CAT reporter plasmids were cotransfected with either the filler plasmid or an activator plasmid. Forty hours after transfection, the cells were harvested for the determination of CAT enzyme activity (9), the isolation of total RNA (22), and the isolation of plasmid DNA (1). The transfections were standardized for variations in transfection

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TABLE 1. Promoter activity in U-2OS and Saos-2 cells[a]

Activator	% Acetylation[b] for extract from promoters in cell lines:					
	SV40 late		RSV LTR		Adenovirus E3	
	U-2OS	Saos-2	U-2OS	Saos-2	U-2OS	Saos-2
Basel	0.6	0.2	4.0	8.1	0.02	0.02
+ T antigen	6.0 (10)	6.5 (34)	38.2 (9.6)	66.4 (8.2)	NT	NT
+ E1a	NT	NT	NT	NT	0.9 (45)	0.9 (45)

[a] Cells were transfected and harvested for CAT enzyme assay as described in the text.

[b] Percent acetylation was determined (from repeated assays) by cutting out and counting the acetylated and unacetylated forms of [^{14}C]chloramphenicol from thin-layer chromatography plates. The results were standardized for variations in transfection efficiencies (see text). Numbers in parentheses are fold activation (CAT activity with activator versus CAT activity with no activator). NT, Not tested.

efficiency by dot blot determination of the relative amount of plasmid DNA in the cells at the time of harvest. This procedure has previously been shown to be an accurate means of standardization (1). Polyadenylated RNA was selected from total RNA and analyzed by the Northern (RNA) procedure with a CAT-specific [^{32}P]RNA hybridization probe. Replication of plasmid DNA was determined by Southern blot analyses by using the differential sensitivities to methylation of the restriction enzymes *Dpn*I and *Mbo*I (1).

Table 1 shows representative results of transfections carried out to determine the extent of T-antigen-mediated transcriptional activation of the SV40 late promoter and the RSV LTR in Rb-positive U-2OS and Rb-negative Saos-2 cells. Both of these promoters have previously been shown to be activated by T antigen in monkey (CV-1) and human (HeLa) cells (1). To test the activation of the SV40 late promoter, the nonreplicative pL2n-CAT plasmid was used. This eliminated plasmid amplification as a cause for increased CAT activity in the presence of T antigen (14). In addition, E1a-mediated transcriptional activation of the adenovirus E3 promoter (1, 23) in each cell was also tested. The data indicate that both the SV40 late promoter and the

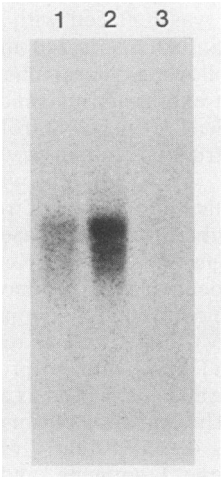

FIG. 1. Northern analysis of polyadenylated RNA derived from transfected Saos-2 cells. Cells were transfected and RNA was extracted and analyzed as described in the text. The following plasmids were used for each transfection: the nonreplicative late promoter CAT plasmid (pL2n-CAT) and the filler plasmid, pSV0-CAT (lane 1); pL2n-CAT and the large-T- and small-t-antigen-producing plasmid, p6-1ΔL (lane 2); pSV0-CAT and p6-1ΔL (lane 3).

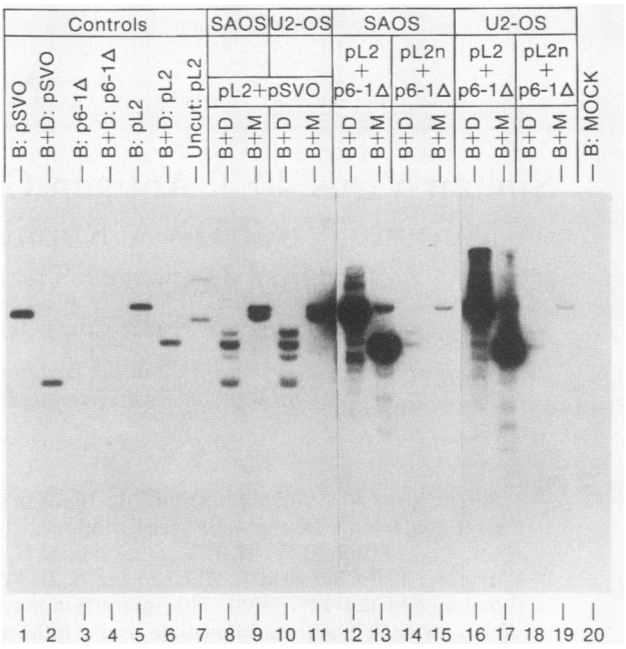

FIG. 2. Southern analysis of T-antigen-mediated replication of plasmids containing the SV40 origin of replication in U-2OS and Saos-2 cells. Cells were transfected and plasmid DNA was harvested and analyzed as described in the text. The lanes are designated by cell type, plasmid(s) transfected, and restriction enzyme used for analysis. B, *Bam*HI; D, *Dpn*I; M, *Mbo*I. The first seven lanes were enzyme cleavage controls with purified plasmids. The hybridization probe was ^{32}P-labeled RNA complementary to CAT gene sequences. All lanes were derived from the same experiment; however, lanes 1 to 11 were derived from a film exposed for 6 h, while lanes 12 to 20 were derived from films exposed between 1 and 2 h. Mock, mock transfected.

RSV LTR were activated equivalently in both cell lines. Eight- to tenfold activation was normally seen with large T antigen. The E1a protein also activated the E3 promoter equivalently in each cell line.

To confirm that the increased CAT activity generated from the SV40 late promoter plasmid was the consequence of increased levels of RNA, polyadenylated RNA was quantitatively selected from total RNA harvested from transfected cells and was analyzed by the Northern procedure with a hybridization probe specific for CAT RNA. Figure 1 shows the results of Northern analysis of the polyadenylated RNA from Saos-2 cells which were mock transfected (lane 3) and transfected with the late promoter plasmid in the absence (lane 1) or presence (lane 2) of T antigen. The intensities of the CAT mRNA bands were determined by laser densitometry; after correction for transfection efficiency (see above), there was a seven- to eightfold increase in CAT-specific RNA in Saos-2 cells cotransfected with the T-antigen-producing plasmid. Similar results were obtained with U-2OS cells (data not shown) and CV-1 (1).

We then tested whether the function of T antigen in viral DNA replication was similar in each cell line. Since the Saos-2 and U-2OS cells are of human origin, they supported the replication of DNA containing the SV40 origin in the presence of T antigen. Figure 2 shows the results of replication analyses with the replicative pL2-CAT and the nonreplicative pL2n-CAT plasmids in the presence or absence of T antigen. Harvested DNA was cleaved first with *Bam*HI, to linearize the plasmids, and then with either *Mbo*I or *Dpn*I.

These enzymes cleave the same sequence but require different states of methylation. *Dpn*I can cleave the methylated plasmid DNA derived from *Escherichia coli*; hence, plasmid DNA which has replicated in eucaryotic cells will not be cleaved by *Dpn*I. Conversely, *Mbo*I cannot cleave methylated plasmid DNA derived from *E. coli*; however, plasmid DNA will become sensitive to *Mbo*I if the DNA has replicated in eucaryotic cells. The DNA was cleaved and analyzed by the Southern procedure with a CAT gene-specific probe; hence, the restriction pattern is simplified, since only restriction fragments which contain CAT sequences will be detected. Controls showing the band patterns for pSV0-CAT, p6-1ΔL, and pL2-CAT (pL2n-cat has the same patterns) were cleaved with *Bam*HI alone or with both *Bam*HI and one of the analytical enzymes (Fig. 2, lanes 1 through 7). Note that no hybridization occurred with p6-1ΔL, because this plasmid contains no CAT sequences. The replicative plasmid pL2-CAT did not amplify in either Saos-2 or U-2OS cells when the T-antigen-supplying plasmid was not provided (i.e., *Dpn*I cleaved the DNA and *Mbo*I did not) (Fig. 2, lanes 8 through 11). Conversely, when the T-antigen-supplying plasmid (p6-1ΔL) was cotransfected, pL2CAT replicated in both cell lines (Fig. 2, lanes 12 and 13 for Saos-2 and lanes 16 and 17 for U-2OS). As expected, the nonreplicative plasmid pL2n-CAT did not replicate in either cell line, regardless of the presence of T antigen (Fig. 2, lanes 14 and 15 for Saos-2 and lanes 18 and 19 for U-2OS).

The data presented in this paper indicate that Rb protein need not be present in cells for the efficient functioning of T antigen in transcriptional activation and viral DNA replication. These data imply that the binding of T antigen to Rb protein is not required for a positive effect on transcriptional activation or the processes leading to viral DNA replication. The data do not rule out the possibility that a class of promoters, different from the SV40 late promoter and the RSV LTR, which is dependent on Rb protein for activation may exist. In addition, the data do not rule out the possibility that the binding of Rb protein by T antigen may eliminate a negative effect. Specifically, if Rb protein negatively controls transcriptional activation and DNA replication, then the binding of Rb protein by T antigen may inhibit this effect, allowing transcriptional activation or the processes leading to replication. However, this possibility can potentially be ruled out, since mutant T antigens which have lost the ability to bind Rb protein (5) but which maintain wild-type levels of viral DNA replication and grow in the same manner as the wild type does in lytic infections have been found (12; for example, mutant K1, which is a Glu to Lys change at amino acid 107). Hence, it appears that the functions of T antigen in transcriptional activation and the processes leading to viral DNA replication are not directly related to the interaction between large T antigen and Rb protein.

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