Stimulation of the Human Heat Shock Protein 70 Promoter in Vitro by Simian Virus 40 Large T Antigen*

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Ian C. A. Taylor‡‡, William Solomon‡‡, Beth M. Weiner‡, Eva Paucha**, Margaret Bradley**, and Robert E. Kingston‡‡

From the ‡Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, the ‡‡Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, and the Departments of **Pathology and Biological Chemistry and Molecular Pharmacology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

Simian virus 40 large tumor (T) antigen stimulates transcription from the SV40 late promoter and some cellular genes. We report here the novel finding that purified T antigen preferentially stimulates transcription from the human heat shock protein 70 promoter in an in vitro transcription system. T antigen is thus capable of stimulating transcription by a process that does not require synthesis of other proteins and that may involve a direct interaction with preexisting cellular factors.

The large T antigen of SV40 possesses multiple functions. T antigen is the first of the "early" genes expressed by the virus after infection, and it helps govern the viral life cycle and virus-cell interactions (for a review, see Ref. 1). For example, T antigen is known to control its own expression by transcriptional repression (2, 3), is dispensable for viral genome replication (2), and stimulates SV40 late gene expression via transcriptional activation ("transactivation"; 4-6). Stimulation of some host genes also has been attributed to T antigen expression (7-9). Although the role of T antigen in viral early gene expression has been well studied, the precise mechanism by which it stimulates transcription has remained elusive.

Biologically active T antigen has been purified, but it has not previously been shown to stimulate transcription in vitro. To test for T antigen function in vitro, we have chosen to use the human hsp70 promoter. While transactivation by T antigen has been studied most carefully in vivo for the SV40 late promoter, this promoter is inefficiently transcribed in vitro, rendering it difficult to study. The human hsp70 promoter used here is induced by E1A upon adenovirus infection (10, 11) as well as by polyoma T antigen (12), c-myc (13), and SV40 T antigen in cotransfection assays (see below), suggesting that it is a reasonable model promoter for transactivation studies. It is well characterized and has significant basal activity both in vivo and in vitro.

We show that transcription of the hsp70 promoter in vitro is dependent on the same sequences that are required in vivo. We further show that addition of purified T antigen preferentially stimulates transcription from this human promoter in vitro. This suggests that this activation is due to protein-protein interactions between T antigen and cellular factors and that T antigen does not increase the absolute level of these factors but instead augments or modifies their activity.

EXPERIMENTAL PROCEDURES

Plasmids—All plasmids were purified by banding twice with ethidium-bromide centrifugation. DNA concentrations were determined spectrophotometrically and verified by agarose gel electrophoresis. Construction of large T antigen expressing plasmid pZIPPvU-0 has been described previously (14). Plasmid pSV-H1 was constructed by removing the BamHI fragment containing the T antigen sequences in pSVH1 (generous gift of P. Jat, Massachusetts Institute of Technology). Plasmids pXGH5 (15), pLR7 (12), and linker scan mutants of the hsp70 promoter (16; Fig. 3) have been described previously.

Transfection Protocol and Human Growth Hormone Assays—Balb/c 3T3 cells were passaged in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum. The calcium phosphate coprecipitation method was used as described elsewhere (13). In transactivation studies, cells were transfected with 6 μg of the hsp70-CAT plasmid, 6 μg of the internal reference plasmid pLR7-184 (16), 1 μg of pXGH5, and 5 μg of the T antigen expressing plasmid or vector control. pXGH5 contains the mouse metallothionein promoter fused to the human growth hormone reporter gene (15) and was included in every experiment as a control for transfection efficiency. We were concerned that T antigen might have some specific effect on MT1-IgH expression, making it less useful as a control for transfection efficiency. However, in 36 separate transfections, T antigen caused on average a 14% reduction in MT1-IgH expression. Experiments were done in both HeLa and Balb/c 3T3 cells; however, we report the results of the Balb/c 3T3 experiments because the degree of transactivation in this cell line was somewhat higher and more consistent that was seen in HeLa cells. The source of this variation in HeLa cells is unknown and was not further pursued. Human growth hormone assays were as described (16).

Infections—HeLa cells were seeded in 60-mm tissue culture dishes. Infections were performed when cells reached a density of 5 × 10⁴ per plate (approximately 50% confluent). Dulbecco’s minimal essential media were removed and saved. Cells were washed once with serum-free Dulbecco’s minimal essential medium and then infected at a multiplicity of infection of 10. Cells and virus were incubated for 1 h at 37 °C after which the virus was removed and the saved medium was replaced. Cells were incubated at 37 °C and harvested for RNA (see below) at various times postinfection.

RNA Preparation and Analysis—Total cellular RNA was obtained by the guanidinium-CsCl method (17). Preparation of the single-stranded human hsp70 probe (containing bases -229 to -133 of the hsp70-CAT fusion gene) has been described (12). Preparation of the human β2-microglobulin probe was by modification of the procedure of Ley et al. (18). The template was a PstI fragment contained in an M13 clone, and the body-labeled probe was synthesized by primer extension. S1 nuclease analysis (19) was performed as described previously (16). A synthetic oligonucleotide (see below) was used as a S1 nuclease probe for the endogenous hsp70 RNA harvested in infection experiments.

In Vitro Transcription—In vitro transcription was performed using a modification of the procedure of Manley et al. (20). Nuclear extracts

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† Present address: Box 55, SUNY-Brooklyn, Brooklyn, NY 11203.

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RESULTS

In order to study the effect of T antigen expression on transcription in vitro, we needed an appropriate target promoter. Previous work demonstrated that SV40 infection of monkey kidney cells results in an increase of expression of a hsp70-like gene product (23), raising the possibility that transcription from the human hsp70 promoter is increased by T antigen. As preliminary experiments to examine stimulation of the human hsp70 promoter by T antigen, we studied the effect of T antigen on this promoter in intact cells using both infection and cotransfection protocols.

HeLa cells were infected by the SV5 strain of SV40 virus at a multiplicity of infection of 10 or were mock-infected. At various times postinfection the cells were harvested for total cellular RNA and the level of appropriately initiated transcripts from the endogenous human hsp70 promoter was determined by S1 analysis. Cellular β2-microglobulin RNA was probed separately as a control. The results in Fig. 1 show that expression of the endogenous hsp70 gene is stimulated approximately 4-fold relative to mock-infected cells between 8 and 12 h postinfection. A decrease in the stimulation is seen at 16 h postinfection; however, this phenomenon was not found to be entirely reproducible in several experiments and its significance here is not known. A further increase in stimulation (6-fold) is seen at 24 h postinfection.

Is this observed stimulation due to T antigen? We cotransfected hsp70 promoter-CAT fusion genes with a plasmid that expressed wild-type T antigen into Balb/c 3T3 cells (Fig. 2). The test hsp70 promoter used was full-length (to −1250) or was deleted to −84 for all heat shock elements, thus eliminating any possibility that the observed stimulation was due to stressing the cell. This deletion also removes all putative T antigen binding sites in the promoter (see “Experimental Procedures”). We performed S1 analysis on total RNA isolated from transfected cells (Fig. 2). Both a test hsp70 promoter and an internal reference hsp70 promoter were cotransformed into HeLa cells by the method of Dignam et al. (21).

Transcription reactions (20 μl) contained 200 ng of supercoiled hsp70 template DNA, 50 ng of p91023 (adenovirus major late promoter, used as an internal reference), 3 mM MgCl2, 2 mM spermidine, 200 ng of poly(dI-dC):poly(dI-dC), 5 μM creatine phosphate, 400 μM each ribonucleotide, and 12 μl of HeLa nuclear extract (4 μg/ml; 48 μg protein) in Dignam buffer D. Transcription was performed at 30 °C for 60 min. Transcription levels under these conditions increased linearly through 120 min of incubation. RNA was analyzed by S1 nuclease analysis as described above. Synthetic oligomers labeled at the 5′ end were used as probes. The human hsp70 oligo probe contains bases +50 to −10 of the hsp70 gene while the adenovirus major late oligo probe contains bases +35 to −6 of the major late gene. Gels were quantitated by excising the bands and counting the slices in a scintillation counter or by film densitometry with a Helena Laboratories Quick Scan R&D densitometer. The relative transcription level of the hsp70 promoter (RTL) was calculated as described below (Fig. 3, legend).

SV40 large T antigen was immunopurified by the method of Simanis and Lane (22). SV40 strain CS1085 was used to infect monkey kidney (CV-1) cells which were harvested for T antigen 48 h later. This strain is a regulatory mutant that makes wild-type large T antigen. Transcription reactions were performed with purified T antigen (approximately 95% pure; Fig. 4B) added to the reaction at various molar amounts in excess of the template DNA. In these reactions, two hsp70 promoter templates were tested, full-length (1250 bases) and truncated (to −105, thus lacking potential T antigen binding sites; see Ref. 12). Transcripts from the hsp70 promoter were analyzed using the longer (+226 to −133) S1 probe described previously (12), while the oligo probe described above was used for the major late transcripts. Increase in RTL due to addition of purified large T antigen was calculated as described below (Fig. 4A, legend).

1 The abbreviation used is: RTL, relative transcription level.
fectected either with the wild-type large T antigen-expressing plasmid pZIP/FVu-0 or the vector control pSV-H1. Message from the internal reference promoter, which contained 84 bases of hsp70 5′-flanking sequence, but a partially deleted 5′-untranslated region, served as an internal control for transactivation by T antigen.

The amount of appropriately initiated transcript from test hsp70 promoters containing both 1250 and 84 bases 5′ sequence was stimulated approximately 3-fold by T antigen (Fig. 2, lanes 1–4). Transcript level from a promoter with a linker scan mutation disrupting the TATA element (mutant 22–26), an element reported to be necessary for stimulation of this promoter by the adenosine Virus E1a products (24), was also stimulated 2–3-fold by T antigen (Fig. 2, lanes 5 and 6). In 27 separate cotransfections, hsp70-CAT expression levels were specifically increased an average of 2.8-fold, with specific stimulation of up to 5-fold observed in certain experiments.

Southern hybridization analysis showed that the observed increase was not caused by stabilization of transfected DNA or by an increase in DNA copy number (data not shown). These data argue that T antigen increases the level of hsp70 transcripts both after infection of human cells and after transfection of the promoter.

Studies on activation of mutant hsp70 promoters in intact cells demonstrated that three separate basal elements of the promoter all play a role in hsp70 expression in both the presence and absence of T antigen (data not shown). We therefore first established that these basal elements were functional in vitro. We programmed the in vitro system with a set of linker scan mutations of the hsp70 promoter (Fig. 3), including mutants in these critical motifs: a CCAAT element at −65, a GC-rich element at −45, and a TATA element at −25 (16, 25, 26). The adenovirus major late promoter was used as an internal control. Mutations in the CCAAT, GC-rich, and TATA elements all decreased transcription in vitro (Fig. 3, lanes 2–4, 7–9, 11–13, respectively). The effects of the mutations in vitro correlate well with their effects in intact cells (16), except that mutation of the TATA homology has a much more severe effect in vitro than in intact cells. This latter observation has been made with several other promoters (27–30).

We next added immunopurified SV40 wild-type T antigen to the in vitro transcription reaction. Preparations of purified T antigen used in these reactions are active as measured by an in vitro replication assay (31; data not shown). Transcription from both a full-length (−1250) and truncated hsp70 promoter (to −105, deleted for all putative T antigen binding sites and thus similar to the truncated hsp70 promoter seen to be stimulated in vivo) was stimulated by T antigen relative to expression from the adenovirus major late promoter (Fig. 4A). There was a highly reproducible 2–4-fold preferential stimulation of each of the human hsp70 promoters under these conditions upon addition of several different preparations of purified SV40 T antigen. T antigen was used at low molar ratios to template DNA (2:51 and 5:1) to limit the possibility that any observed stimulation was caused by an impurity in the T antigen preparation. The purity of the preparation (Fig. 4B) and these low molar ratios argue that the observed effects are due to T antigen; however, we note that we cannot rigorously rule out a role for a contaminating activity.

We were concerned that the observed preferential stimulation of the hsp70 promoter by T antigen may have been an artifact of the precise experimental conditions used. In particular, variations in extract and template concentrations can, in some cases, result in differential effects on transcription of distinct promoters in vitro. Therefore, we examined the extent of T antigen stimulation at various template and extract concentrations (Table I). Preferential stimulation of the human hsp70 promoter relative to the adenovirus major late promoter was seen in all conditions tested. At low extract or low template concentrations, the stimulation was minimal (50% or less) but was still preferential. Boiled T antigen had no stimulatory effect (Table I).

Recent studies on activation of the SV40 late promoter have demonstrated a stable alteration of a cellular transcription factor by T antigen (32). If T antigen catalytically modified some cellular transcription factor in our in vitro experiments, preincubation of T antigen with the extract might increase stimulation. Several different preincubation protocols were tried without observing any increase in the extent of activation (Table II). Indeed, preincubation decreased stimulation.

A stable alteration of a cellular transcription factor by T antigen might be observable in an in vitro binding assay. We and others have identified cellular factors that bind the CCAAT element and the GC-rich element of the human hsp70 promoter (16, 25). Incubation of T antigen with nuclear extracts under conditions that stimulate transcription does not cause any detectable alteration in these factors as analyzed by mobility shift gel electrophoresis (data not shown).
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**Effect of nuclear extract and DNA template concentration on in vitro transactivation by T antigen**

In *vivo* transcription reactions were performed as described under "Experimental Procedures" with the condition changes shown. S1 nuclease gels were quantitated either by densitometry or by counting gel slices in a scintillation counter.

| [HelA nuclear extract] | [DNA template] | Transactivationa |
|------------------------|----------------|-----------------|
| mg/ml                  | mg/ml          | 2.5 x            |
| 0.5                    | 10.0           | 1.2             |
| 1.0                    | 10.0           | 1.2             |
| 1.5                    | 10.0           | 1.8             |
| 3.0                    | 10.0           | 3.0             |
| 1.5                    | 1.0            | 2.1             |
| 1.5                    | 5.0            | 4.6             |
| 1.5                    | 1.5            | 1.4             |

*a Preferential stimulation of the human hsp70 promoter relative to the adenovirus major late promoter, calculated as described in Fig. 4A. 1.0 is the value assigned to the hsp70 major late promoter ratio in the absence of T antigen. "2.5 x" and "5.0 x" represent the molar excess of purified T antigen, over template DNA, that was added to the reaction.

Furthermore, in these same mobility shift assays, purified T antigen could not be detected to bind to promoter DNA lacking the putative T antigen binding sites (12) in the presence or absence of nuclear extract (data not shown).

**DISCUSSION**

The SV40 T antigen can transactivate a variety of promoters, both viral and cellular (4–9, 23). We began an analysis of the mechanism of this transactivation by T antigen by studying activation of the human hsp70 promoter in *vivo*. We chose to study this promoter in *vivo* because the level of cellular human hsp70 RNA is increased upon infection of HeLa cells by SV40 virus. We argue, via cotransfection studies, that T antigen expression is responsible for this increase. We note that these data fall short of rigorously demonstrating that T antigen stimulates transcription from this promoter in *vivo*, as further experiments, such as nuclear run-on in *vivo* transcription experiments under infection conditions, are required before such definitive statements can be made. Despite this caveat, we observe clear effects of T antigen on transcription from this promoter in *vivo*, and we believe that this reflects an important enzymatic property of this protein.

We were interested in whether or not we could observe preferential stimulation of the hsp70 promoter by T antigen in a cell-free system as a first step in determining the mechanism for this transactivation. It was possible that in *vivo*, T antigen transactivates indirectly by increasing the synthesis, and thus the level, of some cellular factor, leading to increased transcription. Instead, we found that T antigen is capable of promoter was calculated as described in Fig. 3. In this case, WT is the signal from the hsp70 promoters without T antigen and L5 is the signal from the hsp70 promoters in the presence of purified large T antigen. RTLV values: no T antigen (lane 1): −1250 = (1.0); −105 = (1.0); 2.5 molar excess T antigen (lane 2): −1250 = 4.6; −105 = 3.0; 5.0 molar excess T antigen (lane 3): −1250 = 3.2; −105 = 2.1. M represents a MspI digest of pBR322 DNA used as size markers. B, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of immunopurified large T antigen. Increasing amounts of immunopurified large T antigen (denoted by arrow) were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel which was then silver-stained (17). Lane 4, 150 ng of purified protein; lane 5, 300 ng; lane 6, 750 ng. Heavy and light chains of IgG proteins from the fractionation is visible (solid circles). We believe that the faint bands flanking T antigen arise during long-term storage of the preparation. M represents size markers whose molecular weights are shown at the right of the figure.
TABLE II
Effect of preincubation conditions on in vitro T antigen transactivation

| Added 1st | Incubation | Added 2nd | Transcription 3rd | Ave. stim. |
|-----------|------------|-----------|-------------------|-----------|
| N.E. + T Ag + DNA | 30 min | 30 °C | rNTPs | 60 min | 2.1 |
| N.E. + boiled DNA + T Ag (5 min) | 30 min | 30 °C | rNTPs | 60 °C | 0.9 |
| DNA + T Ag | 30 min | N.E. | rNTPs | 60 min | 2.2 |
| DNA + N.E. | 30 min | T Ag | rNTPs | 60 min | 1.5 |
| N.E. + T Ag | 45 min | DNA | rNTPs | 60 min | 1.9 |
| N.E. + T Ag | 45 min | | DNA | rNTPs | 60 min | 1.4 |

* Represents the average preferential stimulation (Ave. stim.) of the human hsp70 promoter relative to the adenovirus major late promoter observed with varying molar excess (2.5 to 10 x), over template DNA, of purified T antigen (T Ag) added under each condition. Stimulation, or -fold induction, was calculated as described in Fig. 44.

stimulating hsp70 transcription in vitro, which argues for a more direct transactivation mechanism, involving a protein-protein interaction between T antigen and cellular factor(s). For example, T antigen could increase hsp70 transcription in vitro by directly interacting with components of the cellular transcription machinery, by modifying one of the transcription factors known to be important for hsp70 expression, or by initiating a protein cascade that results in modifying the transcription machinery. A similar activity may play a role in transactivation of other promoters by T antigen.

T antigen shares with the adenovirus Ela proteins the ability to stimulate transcription in the apparent absence of sequence-specific DNA binding to the activated promoter. Ela has been proposed to interact with cellular transcription factors, either to modify their activity, or to bind to the transcription complex and increase its activity (for a review, see Ref. 33). Perhaps Ela and T antigen share a common enzymatic property that results in transactivation. The in vitro system that we report here can be used to address these and other possibilities so as to piece together a more detailed biochemical picture of the precise mechanism by which T antigen transactivates cellular genes.

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REFERENCES
1. Bradley, M. K., and Livingston, D. M. (1982) Mol. Biol. Med. 4, 63-80
2. Tjian, R. (1981) Cell 26, 1-2
3. Rio, D., Robbins, A., Myers, R., and Tjian, R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5706-5710
4. Alwine, J. C. (1985) Mol. Cell. Biol. 5, 1034-1042
5. Brady, J., and Khoury, G. (1985) Mol. Cell. Biol. 5, 1391-1399
6. Keller, J. M., and Alwine, J. C. (1984) Cell 36, 381-389
7. Lane, D. P., Simian, V., Bartosh, R., Yewdell, J., Gannon, J., and Mole, S. (1985) Proc. R. Soc. Lond. B Biol. Sci. 226, 25-42
8. Rigby, P. W. J., La Thangue, N. B., Murphy, D., and Skene, B. I. (1985) Proc. R. Soc. Lond. B Biol. Sci. 226, 15-23
9. Schutzbank, T., Robinson, R., Oren, M., and Levine, A. J. (1982) Cell 30, 481-490
10. Kao, H.-T., and Nevins, J. R. (1983) Mol. Cell. Biol. 3, 2058-2065
11. Nevins, J. R. (1982) Cell 30, 913-919
12. Kingston, R. E., Cowie, A., Morimoto, R. I., and Gwinn, K. A. (1986) Mol. Cell. Biol. 6, 3180-3190
13. Kaddurah-Daouk, R., Greene, J. M., Baldwin, A. S., Jr., and Kingston, R. E. (1987) Genes Dev. 1, 347-357
14. Kaidorn, D., and Smith, A. W. (1984) Virology 139, 109-137
15. Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M., and Moore, D. D. (1986) Mol. Cell. Biol. 6, 3173-3179
16. Greene, J. M., Larin, Z., Taylor, I. C. A., Prentice, H., Gwinn, K. A., and Kingston, R. E. (1987) Mol. Cell. Biol. 7, 3646-3655
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology, Greene Publishing Associates/Wiley-Interscience, New York
18. Ley, T. J., Anagou, N. P., Pepe, G., and Nienhuis, A. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4775-4779
19. Berk, A. J., and Sharp, P. A. (1977) Cell 12, 721-732
20. Manley, J. L., Fire, A., Samuels, M., and Sharp, P. A. (1983) Methods Enzymol. 101, 568-582
21. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1484
22. Simian, V., and Lane, D. P. (1985) Virology 144, 88-100
23. Khandjian, E. W., and Turler, H. (1983) Mol. Cell. Biol. 3, 1-8
24. Simon, M. C., Fisch, T. M., Benecke, B. J., Nevins, J. R., and Heintz, N. (1988) Cell 52, 723-729
25. Morgan, W. D., Williams, G. T., Morimoto, R. I., Greene, J. M., Kingston, R. E., and Tjian, R. (1987) Mol. Cell. Biol. 7, 1129-1138
26. Ku, B., Hunt, C., and Morimoto, R. (1985) Mol. Cell. Biol. 5, 330-341
27. Gordon, J., Wasylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C., and Chambon, P. (1980) Science 209, 1406-1414
28. Grosschedl, R., and Birnstiel, M. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 297-301
29. Proudfoot, N. J., Shander, M. H. M., Manley, J. L., Geifer, M. L., and Maniatis, T. (1980) Science 209, 1329-1336
30. Taunimoto, Y., Hirose, S., Tsuda, M., and Suzuki, Y. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4838-4852
31. Li, J. J., and Kelly, T. J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6973-6977
32. Gallo, G. J., Gilinger, G., and Alwine, J. C. (1988) Mol. Cell. Biol. 8, 1645-1656
33. Kingston, R. E., Baldwin, A. S., and Sharp, P. A. (1985) Cell 41, 3-5