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Binding of Polyomavirus Large T Antigen to the Human *hsp70* Promoter Is Not Required for *trans* Activation

ROBERT E. KINGSTON,[1,2]* ALISON COWIE,[3] RICHARD I. MORIMOTO,[4] AND KATRINA A. GWINN[1,2]

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114[1]; Department of Genetics, Harvard Medical School, Cambridge, Massachusetts 02115[2]; Genetics Institute, Cambridge, Massachusetts 02140[3]; and Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60201[4]

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Polyomavirus large T antigen binds to two sites located between positions −110 and −170 of a human heat shock protein 70 (hsp70) promoter. Methylation interference studies show that binding for each site is determined by two GPuGGC pentanucleotide sequences. The specificity of this binding interaction is similar to that observed for large T binding to the viral genome. The existence of sequences that bind a viral protein in a cellular promoter raises the possibility that these sequences play a role in gene expression in an uninfected cell. We show that hsp70 large T antigen binding site 1 is capable of functioning as an upstream promoter element in cells that do not contain any viral T antigen. Genetic analysis of this effect suggests that a cellular factor exists that has a binding specificity that overlaps but is not identical to that of polyomavirus large T antigen. To determine whether binding of polyomavirus large T antigen can regulate expression of the intact human hsp70 promoter, we have introduced the promoter into mouse cells with plasmids that express the polyomavirus early proteins. These proteins stimulate the level of correctly initiated hsp70 transcripts, but surprisingly the degree of stimulation remains unchanged for promoter constructs in which the large T antigen binding sites have been deleted. These observations suggest that *trans* activation of the hsp70 promoter by the polyomavirus early proteins occurs through protein-protein interactions and not through sequence-specific DNA binding.

Polyomavirus, simian virus 40 (SV40), and adenovirus are all double-stranded DNA tumor viruses which encode proteins capable of establishing primary cells in culture. For each virus the genes responsible for this immortalization function have been identified (25, 45, 48). In all cases the gene products are nuclear antigens. Polyomavirus large T and SV40 large T antigens are sequence-specific DNA-binding proteins (20, 52, 53). Both the adenovirus E1a region and SV40 large T antigen have been shown to be capable of stimulating transcription in *trans* from viral promoters as well as from some transfected cellular promoters (1, 4, 6–8, 21, 24, 30, 32, 33). Other viruses capable of establishing primary cells in culture contain genes that encode nuclear proteins that share this ability to *trans* activate promoters, leading to the hypothesis that an ability to regulate cellular promoters is necessary for immortalization activity (reviewed in reference 36). One prediction of this hypothesis is that immortalizing gene products will directly interact with cellular promoter regions to either repress or activate those promoters.

One promoter that immortalizing gene products might regulate is the human heat shock protein 70 (hsp70) promoter. The promoter is activated during adenovirus infection, and *hsp70* gene expression is elevated in some tumor cell lines as well as in SV40-infected cells (27, 31, 34, 43, 57a). The c-*myc* gene product, a cellular protein capable of immortalizing cells, is capable of stimulating expression from a *Drosophila melanogaster hsp70* promoter region (35). In addition, the hsp70 protein is strongly conserved from bacteria to humans, suggesting that it plays a fundamental role during normal cellular growth (2, 26). Deletion of this gene in *Saccharomyces cerevisiae* results in impaired growth at normal temperatures, implying that this protein has a function independent of its role in protecting cells from stress (13, 14).

There are several loci that encode the hsp70 protein or related proteins in all eucaryotic organisms characterized to date. The human genome encodes at least five loci. The coding region of the human gene studied in this work has a high degree of homology to both the *Drosophila hsp70* gene and to *Escherichia coli dnaK*, identifying it as a member of the *hsp70* family (26, 57). Although there is a high constitutive level of the mRNA for this gene in HeLa cells, RNA levels remain heat inducible. The promoter contains a sequence between −93 and −107 that is homologous to the consensus heat shock regulatory sequence found in *Drosophila hsp70* promoters. Deletion analysis is consistent with this element alone being responsible for heat inducibility (58). The promoter is induced when fresh serum is added to resting cells (59), and the sequences responsible for serum stimulation lie within 58 nucleotides of the transcription start site (58).

We have determined the ability of the polyomavirus early region proteins to interact with this human *hsp70* promoter. The polyomavirus early region encodes three proteins, termed large, middle, and small T antigens. Large T antigen is capable of immortalizing primary cells in culture, while middle T antigen is required for full transformation of cells (47, 48, 54). We demonstrate here that large T antigen is capable of binding to two sites in the human *hsp70* promoter. The proteins of the polyomavirus early region are capable of stimulating expression of a cloned *hsp70* promoter in *trans*,

* Corresponding author.

but this stimulation is not dependent on the presence of the large T antigen binding sites.

MATERIALS AND METHODS

Purification of polyomavirus large T antigen. Polyomavirus large T antigen was purified from mouse 3T6 fibroblasts infected with the wild-type A2 strain as previously described (16). The large T antigen thus obtained was dialyzed against binding buffer (20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 7.2, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT]) containing 10% (vol/vol) glycerol. Protein concentration was estimated by silver stained sodium dodecyl sulfate (SDS)-acrylamide gels with known amounts of protein size markers (BioRad) as a standard. Bovine serum albumin (Boehringer) was added to 100 μg/ml before storage in liquid nitrogen. Typically the concentration of T antigen was 1 to 5 μg/ml.

DNA immunoprecipitation binding assays. Plasmid DNAs were cut with *Hin*dIII-*Bam*HI, *Hin*dIII-*Bam*HI-*Nco*I, or *Hin*dIII-*Eco*RI. Each digest (100 ng) was 3′-end labeled with T4 DNA polymerase (P-L Biochemicals) and [α-^{32}P]dCTP (>5,000 Ci/nmol; NEN) (44). Portions (10 ng) of each labeled digest were incubated wtih 20 μl of purified T antigen is 0.5 ml of binding buffer (20 mM PIPES, pH 7.2, 0.1 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μg of bovine serum albumin [BSA] per ml) additionally containing 0.1% Nonidet P-40 (NP40) and 5 μg of salmon sperm DNA per ml. The bound DNA fragments were immunoprecipitated, eluted, and analyzed by agarose gel electrophoresis as previously described (11).

For the binding affinity comparison assays, specific-3′-end-labeled DNA fragments were first isolated by agarose gel electrophoresis and eluted. The purified DNA fragments were incubated with increasing amounts of partially purified large T antigen in a final volume of 100 μl of binding buffer either with or without salmon sperm DNA (5 μg/ml) added. The fraction of DNA bound was separated by immunoprecipitation as before and the two fractions were counted.

DNase I footprinting. To generate 5′-end-labeled DNA fragments containing the promoter-proximal binding sites, pΔ1250 DNA was digested with *Nco*I, phosphatased, and labeled by [γ-^{32}P]ATP (>3000 Ci/mmol) and polynucleotide kinase (P-L Biochemicals) and then digested with *Pst*I, and the resulting fragment was isolated by nondenaturing polyacrylamide gel electrophoresis. For the opposite strand, pΔ1250 was first digested with *Sac*I, 5′ end labeled, and recut with *Nco*I, and the fragment was isolated as before.

Protection from partial DNase digestion of labeled DNA fragments bound by polyomavirus large T antigen was examined by a modification of the method of Galas and Schmitz (19). Known amounts of 5′-end-labeled DNA fragment (1 to 2 fmol) were incubated with 0, 10, or 25 μl of partially purified large T antigen in a final volume of 100 μl of binding buffer (20 mM PIPES, pH 7.2, 0.1 M NaCl, 1 mM DTT, 1 mM EDTA, BSA [100 μg/ml]) at 20°C for 30 min. Partial DNase I digestion was done by addition of $MgCl_2$ to 5 mM, $CaCl_2$ to 1 mM, and DNase I (Miles-Yeda) to a final concentration of 5 ng/ml and incubation for a further 10 min at 20°C. The reaction was terminated by addition of an equal volume of 2 M ammonium acetate–100 mM EDTA–100 μg of sheared salmon sperm DNA per ml. The samples were extracted with TE-saturated phenol and precipitated from ethanol. They were then dissolved in formamide dye mix (41) and run on 8% polyacrylamide-urea denaturing gels. The gels were dried prior to autoradiography.

Methylation interference. The same 5′-end-labeled double-stranded DNA fragments as were used in the DNase I protection studies were treated with dimethyl sulfate (DMS) as described by Maxam and Gilbert (41). Following ethanol precipitation, the partially methylated fragments were dissolved at a concentration of 1 nM in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (TE). For the large T antigen binding reaction, approximately 1 to 2 fmol of fragment was incubated with 0 to 5 μl of purified large T antigen in a final volume of 100 μl of binding buffer for 30 min at 25°C. To separate the fraction of DNA bound by the large T antigen, 5 μl of tissue culture supernatant of the two monoclonal antibodies αPyLT1 and αPyLT4 (17) were added for 30 min at 25°C. The complexes were precipitated by addition of 20 μl of Pansorbin (Calbiochem) and incubated for 20 min at 25°C. Following precipitation, the bound DNA was eluted from the pellet with 200 μl of TE and 1% SDS. The protein and *Staphylococcus aureus* A bacteria were removed by phenol extraction, and the DNA was recovered by addition of ammonium acetate to 0.5 M and ethanol precipitation. The two DNA fractions were then treated with 1 M piperidine at 90°C for 30 min to cleave the DNA at the methylated guanines. The DNA was recovered as described by Maxam and Gilbert (41) and then analyzed on a 12% polyacrylamide–50% urea denaturing gel. The gels were dried prior to autoradiography. The two monoclonal antibodies αPyLT1 and αPyLT4 were a generous gift from B. Griffin. All autoradiographs were analyzed by densitometer to confirm the presence or absence of each band in each lane.

Transfection protocol. BALB/c 3T3 cells were passaged in DME supplemented with 10% calf serum. Calcium phosphate precipitates were prepared by a modification of the procedure of Graham and van der Eb (23). DNA (10 to 12 μg total) was ethanol precipitated and suspended in 0.5 ml of 250 mM $CaCl_2$. A 0.5-ml amount of 2× HeBS was bubbled while the DNA solution was added dropwise. The precipitate was immediately vortexed for 5 min and allowed to sit at room temperature for 20 min. This solution was then added to a 10-cm dish of cells at 20% confluence that had been fed with fresh DME 2 h earlier. After 16 h, the cells were washed with phosphate-buffered saline and fed with fresh medium. Cells were harvested for either RNA or protein 36 to 48 h after removing the precipitate.

Plasmids. All plasmid DNAs were purified by banding twice with ethidium bromide-CsCl gradient centrifugation. DNA concentrations were determined spectrophotometrically and were verified by agarose gel electrophoresis. Construction of the 5′ deletion series into the human *hsp70* promoter (pΔ plasmids) has been described previously (58), as has plasmid pXGH5 (51a). Other constructions were as follows. pyLT and py3T were the generous gift of Parmjit Jat (28). They were constructed by insertion of a *Bgl*I-*Bam*HI fragment (nucleotides [nt] 87–4633 of polyomavirus) containing either a wild-type polyomavirus early region or a large T cDNA (48) into the unique *Bam*HI site of pZipNeoSV(X)I (9).

Plasmid pIR17 (John Greene, this laboratory) was made by partial digestion of plasmid pΔ1250 with *Pst*I, isolation of the resultant linears, resection with Bal31, and ligation. The plasmid contains a 33-bp deletion extending from +65 to +98 of the *hsp70* 5′ untranslated region.

Plasmids in the pΔ58+ series were made by opening the plasmid pΔ58 with *Hin*dIII and ligating the appropriate nonphosphorylated 25-mer duplex at a 100:1 molar excess. The resultant ligation was diluted to a DNA concentration of

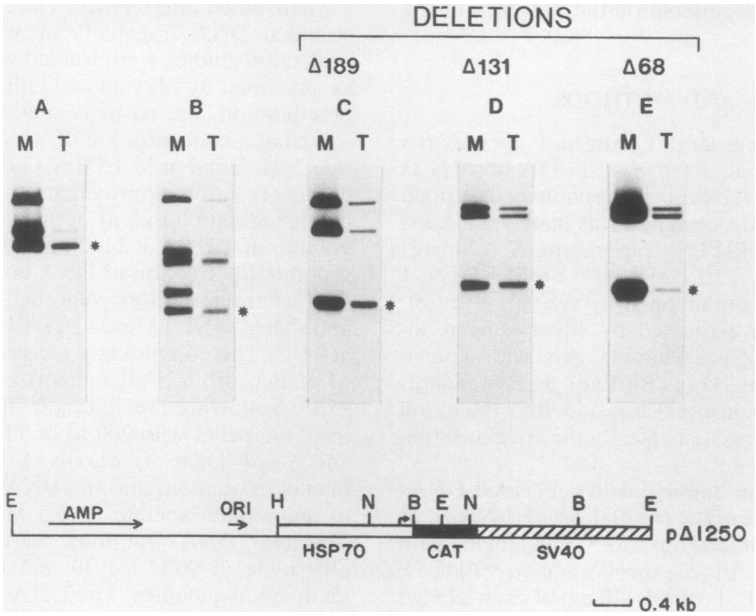

FIG. 1. DNA immunoprecipitation of *hsp70* promoter fragments. ^{32}P-labeled DNA fragments were incubated with purified polyomavirus large T antigen (20 μl) in 0.5 ml of binding buffer containing 5 μg of nonspecific competitor DNA per ml. The DNA fragments bound by large T were immunoprecipitated from the reaction with monoclonal antibodies and then fractionated on a 2% agarose gel (lanes T) with 1/10th the input digest as marker (lanes M). (A) *Hin*dIII (H) plus *Bam*HI (B) digest of pΔ1250. (B) The same DNA digested with *Nco*I (N) in addition to *Hin*dIII and *Bam*HI. (C) pΔ189 digested with *Hin*dIII and *Bam*HI. (D) pΔ131 digested with *Hin*dIII and *Eco*RI (E). (E) pΔ68 digested with *Hin*dIII and *Eco*RI. Positions of restriction enzyme sites with respect to the *hsp70* promoter are shown below. Asterisks mark the promoter-containing fragment.

1 μg/ml, heated at 65°C for 5 min to melt inappropriately hybridized oligonucleotides, and slowly cooled to promote recircularization before transfer into *E. coli*.

All *hsp70* promoter deletions and variants were sequenced by the primed synthesis method (50). Templates for sequencing were prepared by irreversible alkali denaturation of the double-stranded plasmids (10).

RNA preparation and analysis. Total cellular RNA was prepared by the guanidinium-CsCl method (39, 55). Single-stranded end-labeled probes were prepared as follows. A single-stranded 20-mer (0.1 μg) homologous to the coding sequence of chloramphenicol acetyltransferase (CAT; CAA-CGG-TGG-TAT-ATC-CAG-TG) was kinased with ^{32}P and hybridized to single-stranded DNA (20 μg) of a plasmid containing the promoter region and CAT gene of plasmid pΔ133 (10 mM Tris, pH 8.0, 10 mM $MgCl_2$; 45°C for 15 min). The resultant hybrid was extended with Klenow fragment (4 U) and cold deoxytriphosphates (0.4 mM each) at 37°C for 30 min. The extension reaction was stopped by heating at 65°C for 5 min, the buffer was adjusted, and the double-stranded DNA was digested with *Hin*dIII. The resultant fragments were separated as single-stranded DNA by denaturing alkaline agarose gel electrophoresis (39) with low-melting-temperature agarose. The slower-migrating of the two radioactive species contained ^{32}P-end-labeled single-stranded DNA containing bases +229 (labeled) to −133 of the *hsp70-CAT* fusion gene of the pΔH series.

S1 nuclease analysis (4) was performed as follows. From 20 to 40 μg of total cellular RNA was mixed with 3×10^4 dpm of probe, ethanol precipitated, and suspended in hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide). Hybridization was at 30°C for 16 h. S1 nuclease digestion and analysis on 8% denaturing polyacrylamide gels was by standard procedures (39).

Determination of CAT and growth hormone expression levels. CAT extracts were prepared and assayed by standard procedures (22). At the time of harvest for CAT assay, medium was removed from the dishes for determination of growth hormone levels. These samples were assayed by a sandwich radioimmunoassay assay with a commercially available kit (Tandem). Growth hormone levels were within the linear range of the assay as determined by using premixed standards.

RESULTS

Large T antigen binding sites. We were prompted to evaluate the ability of polyomavirus large T antigen to bind to the human *hsp70* promoter by initial observations that the polyomavirus early region could *trans* activate the promoter in cotransfection assays (see below). We investigated binding with plasmid pΔ1250, which contains bases −1250 to +160 of this promoter (Fig. 1). An end-labeled restriction digest of this plasmid was incubated with purified polyomavirus large T antigen, and DNA that bound this antigen was precipitated with monoclonal antibody against large T antigen. A fragment containing sequence from +160 to −1250 of the human *hsp70* promoter was specifically immunoprecipitated (Fig. 1, lane A). This 1.4-kb fragment was cleaved into two fragments by *Nco*I. Analysis of binding following an *Nco*I digest demonstrated that fragments containing either +160 to −320 or −320 to −1250 both contained T antigen binding sites (lane B). We focused on the promoter-proximal binding sites. Deletions extending into the *hsp70* promoter from the 5′ side were used to further localize these sites. There was preferential precipitation of the promoter-containing DNA fragment when deletions to −189 and −131 were tested (lanes C and D), while a deletion to −68

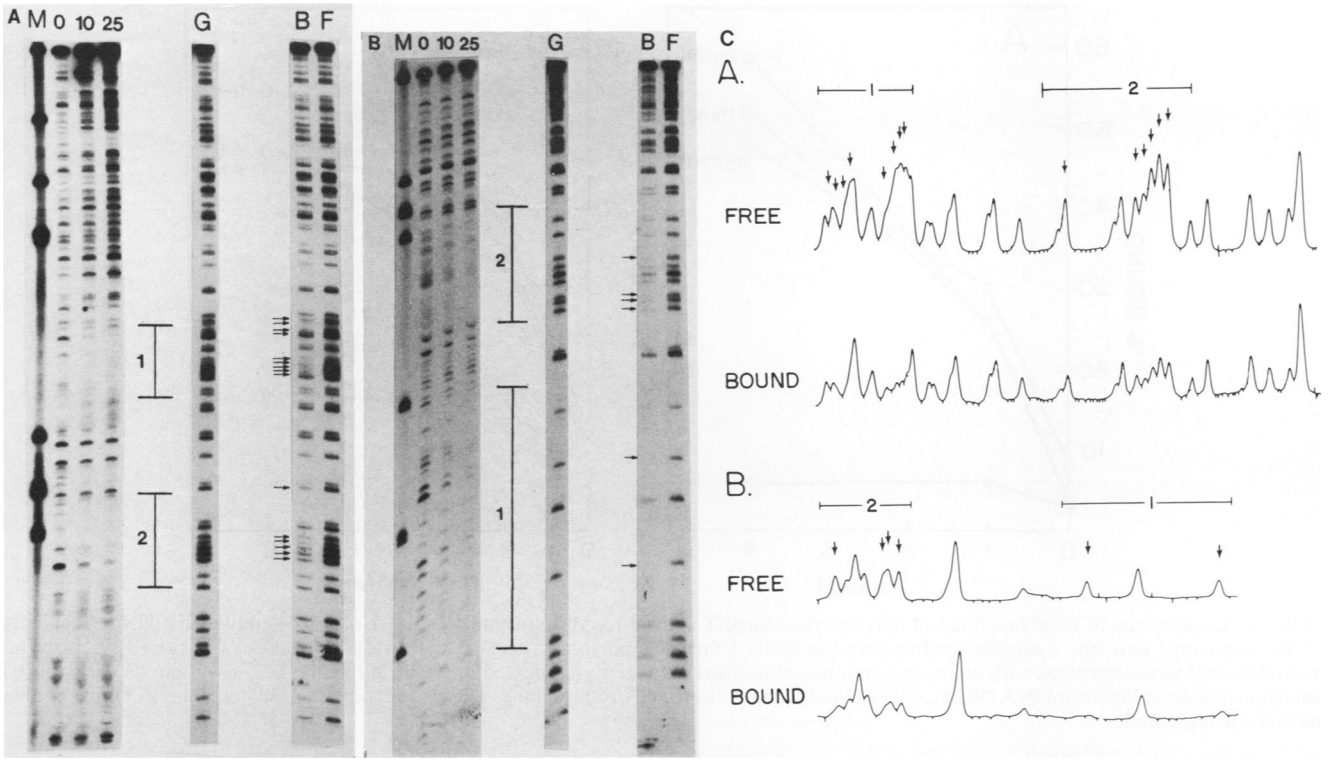

FIG. 2. DNase I footprinting and methylation interference of large T antigen binding to the human *hsp70* promoter region. (A) Left panel: DNA fragment from pΔ1250 labeled at the *Nco*I site was incubated with 0, 10, and 25 μl of large T antigen prior to partial DNase I digestion. The resulting fragments fractionated on an 8% polyacrylamide–50% urea gel together with a size marker (M) and a portion of a DMS cleavage reaction of the same fragment (G). The two regions protected from digestion are indicated by 1 and 2. Right panel: The same 5′-end-labeled DNA fragment was treated with DMS under limiting conditions and then incubated wtih 2 μl of T antigen and the fraction of the DNA bound separated by immunoprecipitation. The cleavage reaction was completed and the bound (B) and free (F) fractions were electrophoresed through an 8% polyacrylamide–50% urea gel. Bands that are underrepresented in the bound fraction are indicated by arrows. (B) Left panel: DNA fragment from pΔ1250, 5′ end labeled on the opposite strand at a *Sac*I site (at −75) was incubated with large T antigen as in panel A. Right panel: The same DNA fragment, 5′ end labeled at the *Sac*I site, treated with DMS as in panel A. (C) Densitometer scans of the DMS interference shown in panel A (A) and panel B (B). Free and bound lanes are marked. The gain on the densitometer was adjusted to normalize for peak heights in comparing the bound and free lanes. Arrows indicate peaks preferentially reduced in the bound fraction.

significantly diminished this interaction (lane E). Inspection of the sequence between −189 and −68 revealed four copies of the sequence GPuGGc. This sequence is known to specify binding to polyomavirus large T antigen (12, 46). We wished to more accurately characterize the T antigen binding interaction to determine whether in this instance it was specified by these sequences.

DNase I protection studies and methylation interference studies were used to further define the large T binding sites. End-labeled DNA fragments spanning the promoter-proximal region were incubated with various amounts of purified polyomavirus large T antigen and subjected to limited DNase digestion. Two separate sites were protected from DNase digestion (Fig. 2A and B, left panels). This protection was seen when either DNA strand was end labeled and used as probe. These sites were located between bases −110 and −170 of the promoter and are referred to as sites 1 (promoter proximal) and 2 (Fig. 3).

Guanine N-7 methylation in the T antigen recognition sequence results in inhibition of the binding of either SV40 or polyomavirus large T antigen to binding sites in their respective viral genomes (12, 29). To characterize the human *hsp70*

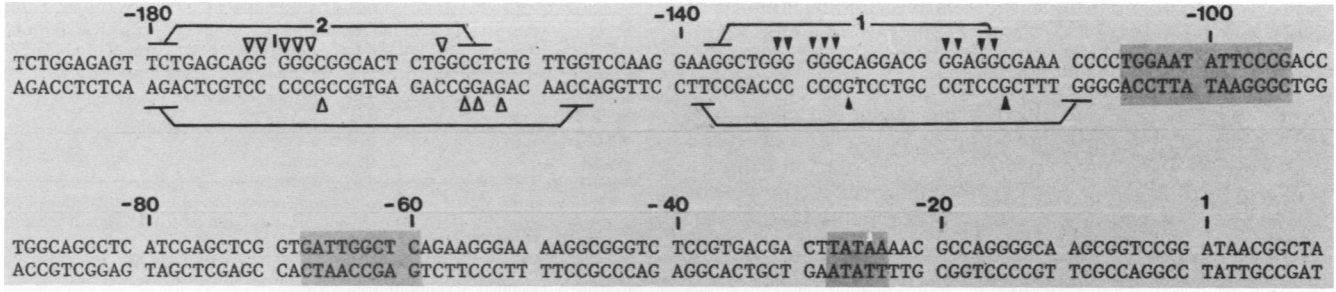

FIG. 3. DNA sequence of the human *hsp70* promoter region (26). The start point for transcription is labeled 1. Brackets summarize the extent of the DNase protection seen in Fig. 2; the triangles summarize the quantitation of the DMS interference from Fig. 2. Solid triangles, Greater than 50% reduction in bound fraction; open triangles, approximately 50% reduction.

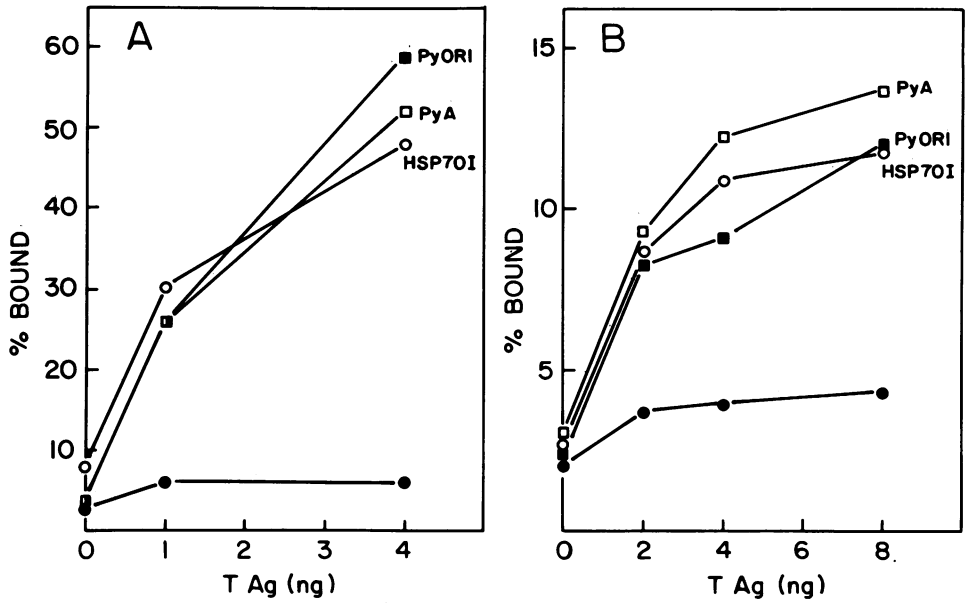

FIG. 4. Comparison of relative affinity of polyomavirus large T antigen for various binding sites. Isolated 5'-end-labeled DNA fragments (0.5 ng) containing only one T antigen binding site: *hsp70* site 1 (hsp70I), polyomavirus site A (PyA), polyomavirus Ori site (PyORI), or no site (unlabeled) were incubated with increasing amounts of purified T antigen as indicated in 100 μl of binding buffer without (A) or with (B) added nonspecific competitor DNA (50 ng). All labeled fragments were 400 to 500 bp long. The amount bound is the percentage of input counts that were precipitated.

binding sites, end-labeled DNA fragments containing both sites 1 and 2 were partially methylated and incubated with purified polyomavirus large T antigen, and the bound and free DNA segments were separated by monoclonal antibody precipitation as described above (Fig. 2). Differences in the intensities of bands in the bound and free fractions were quantitated by scanning densitometry (Fig. 2C). There were 11 G residues in *hsp70* site 1, whose methylation resulted in decreased binding of polyomavirus large T antigen. We observed only minimal effects of methylation in site 2. These data suggest that binding site 1 has a somewhat higher affinity for the protein than binding site 2. DNA impaired for binding in site 2 would be precipitated due to occupancy of site 1, while DNA impaired for binding in site 1 would not be as likely to be precipitated due to occupancy of site 2. The DNase protection and methylation interference results are summarized in Fig. 3. Binding sites 1 and 2 both contained two copies of the pentanucleotide sequence GPuGGC previously shown to specify large T antigen binding. In site 1 these pentanucleotides were in direct orientation and separated by 7 base pairs (bp), while in site 2 the sequences with the best fit to the pentanucleotide sequence were in inverted orientation and separated by 9 bp.

We wished to quantitate the relative binding affinity of *hsp70* site 1 for large T antigen. Using the immunoprecipitation procedure described above, we compared the binding affinity of T antigen for purified DNA fragments containing either 21 bases of *hsp70* site 1 (from plasmid pΔ58+1, see Fig. 7), the polyomavirus site A, or the polyomavirus *ori* site (11). The DNA fragments containing these sites all behaved similarly in this assay both in the absence of competitor DNA (Fig. 4A) and in the presence of competitor DNA (Fig. 4B). These results argue that the relative binding affinity of polyomavirus large T antigen for *hsp70* site 1 is similar to that of T antigen for individual sites in the polyomavirus genome.

***trans* activation by polyomavirus T antigens.** SV40 large T antigen has been shown to stimulate transcription from the SV40 late promoter as well as from several heterologous promoters (1, 6, 32). Stimulation of the late promoter appears to be partly the result of T antigen binding to sequences in the SV40 origin, approximately 250 bp from the late transcription start sites (7, 8, 33). These observations led us to investigate the ability of the polyomavirus early region to *trans* activate the human *hsp70* promoter. To facilitate investigation of the regulation of this promoter, a plasmid was made containing bases −1250 to +160 of the *hsp70* promoter region fused to the *CAT* gene (pΔ1250).

Polyomavirus early region proteins were expressed by using a construction containing the entire early region under control of the Maloney virus long terminal repeat (LTR) (py3T) (P. Jat, unpublished [9]). Cotransfection of pΔ1250 with py3T resulted in a 5- to 20-fold stimulation of CAT activity (Tables 1 and 2; see also Fig. 5). To determine the sequence requirements for this effect, we investigated stimulation of a series of mutant plasmids containing various amounts of 5' flanking sequence. The activity from a deletion

TABLE 1. Stimulation of mutant *hsp70* promoters by the polyomavirus early proteins

Plasmid	Relative basal activity[a]	CAT activity stimulation (fold)[b]
pΔ1250	1.0	8
pΔ131	1.1	9
pΔ68	1.0	5
pΔ58	0.2	10

[a] Basal values are from experiments in which the indicated plasmid (8 μg) was transfected into BALB/c 3T3 cells with an internal control plasmid (pXGH5, 2 μg). The level of CAT activity from each transfection was corrected for the level of growth hormone activity. The activity of pΔ1250 was arbitrarily assigned a value of 1.0.

[b] Stimulation refers to the increase in CAT activity resulting from cotransfecting the indicated plasmid with plasmid py3T (1 μg). Values are from representative experiments repeated at least three times.

TABLE 2. Stimulation of the human *hsp70* promoter region by polyomavirus early proteins[a]

Expt	*hsp70* plasmid	T antigen plasmid (μg)	Stimulation of CAT activity (fold)	Relative stimulation
1	pΔ1250	py3T (0.5)	5	2.9
	pΔ1250	py3T (1)	7	2.7
	pΔ1250	pyLT (0.5)	3	1.3
	pΔ1250	pyLT (1)	3	1.7
2	pΔ1250	py3T (1)	8	2.3
	pΔ131	py3T (1)	19	1.8
	pΔ107	py3T (1)	15	2.1

[a] BALB/c 3T3 cells were transfected with the indicated plasmid (8 μg) and pXGH5 (2 μg). The level of CAT activity obtained with no additional plasmid was compared with that obtained after cotransfection with the indicated polyomavirus early protein expression plasmid. Relative stimulation refers to the degree of stimulation observed after dividing the CAT activity value by the amount of growth hormone produced by the standard amount of pXGH5 included in each transfection. Values are from representative experiments. In six experiments involving 1.0 μg of py3T, CAT stimulation varied from 7- to 19-fold (average of 12-fold) and relative stimulation varied from 1.3 to 2.7 (average, 2.1). In four experiments involving 1.0 μg of pyLT, CAT stimulation varied from 2- to 6-fold (average of 3-fold), and relative stimulation varied from 0.9 to 1.7 (average, 1.4).

that retained only 58 bp upstream of the *hsp70* initiation site was still significantly stimulated by cotransfection with py3T (Table 1). This deletion, while impaired in basal activity, retained the ability to promote transcription from the appropriate initiation site (see Fig. 7) (58). These data argue that the T antigen binding sites are not necessary to observe stimulation. Due to the inherent variability of these experiments, however, they do not rule out a role for the T antigen binding sites in allowing maximal stimulation.

To address the issue of whether the T antigen binding sites contribute to stimulation of this promoter, we included a uniform amount of a reference plasmid (pXGH5) in each further experiment to control for transfection efficiency. Plasmid pXGH5 contains a human growth hormone gene fused to a mouse metallothionein I promoter region (51a). Transfected cells secreted growth hormone into the media, and the amount was easily quantitated through use of a radioimmunoassay. Cotransfection with py3T increased both growth hormone expression from pXGH5 and CAT expression from pΔ1250. The level of CAT activity rose two- to threefold more than the level of growth hormone activity, suggesting that there is some preferential stimulation of the human *hsp70* promoter relative to the mouse metallothionein I promoter (Table 2). Stimulation of CAT expression relative to stimulation of growth hormone expression remained constant as the two T antigen binding sites were removed (Table 2). These data suggest that stimulation of this promoter by the polyomavirus early region is largely independent of any sequence-specific binding of large T antigen.

We sought to determine the start site for *hsp70* transcription in the presence and absence of the polyomavirus early region and to get a second determination of the role of T antigen binding sites in the observed stimulation. We therefore measured the level of correctly initiated transcripts from the *hsp70* promoter in the presence and absence of polyomavirus early proteins. To control for the amount of *trans* activation in this experiment, we used a marked plasmid (pIR17) that contained a wild-type *hsp70* promoter region with a 33-bp deletion in the 5′ untranslated region of the mRNA (Fig. 5). When pIR17 was transfected into BALB/c 3T3 cells and the resulting RNA was analyzed by S1 nuclease analysis with a probe from an unaltered *hsp70-CAT* plasmid, a band of 129 bases resulted due to looping of the probe sequences opposite the deletion in the pIR17 message (Fig. 5, lane A). All of the plasmids containing deletions into the 5′ flanking sequence of the *hsp70* promoter gave rise to an unaltered mRNA, which resulted in a band of 230 bases after S1 nuclease analysis (lane B). It was therefore possible to directly compare the degree of *trans* activation of these promoters with the *trans* activation of the promoter of pIR17 in a single experiment. By this analysis, the wild-type promoter (pΔ1250), a promoter containing only binding site 1 (pΔ133), and a promoter with no T antigen binding sites (pΔ107) were stimulated to an identical degree after cotransfection with py3T in comparison to the wild-type promoter of pIR17 (Fig. 5). This analysis also indicates that the level of mRNA observed correlated with the level of CAT activity and that this mRNA had the appropriate 5′ end in both the presence and absence of *trans* activation.

The polyomavirus early region encodes three proteins: large, middle, and small T antigens. We wished to determine whether large T antigen alone was responsible for the observed stimulation. A plasmid expressing only large T antigen (pyLT) was identical to py3T except that it contained the large T antigen cDNA in place of the entire early region (28, 48). When pyLT replaced py3T in cotransfection experiments, the degree of stimulation of the *hsp70* promoter was lower. These results were obtained when expression was measured by either protein expression or RNA level, and pΔ1250 was stimulated only 1.5-fold more than pXGH5 (Table 2 and Fig. 5). These data suggest that polyomavirus large T plays a role in *trans* activation of the human *hsp70* promoter but may not be entirely responsible for this effect.

T antigen binding site 1 functions as an upstream promoter element. The presence of T antigen binding sequences in a cellular promoter suggested the possibility that these sequences may be part of an upstream promoter element. Deletion of these sequences did not significantly affect *hsp70* promoter activity in BALB/c 3T3 cells (Fig. 5 and 7). This sequence is therefore not necessary for full basal levels of expression from the promoter when the promoter is contained on transfected DNA. However, deletion of the sequence 5′-GATTGGCTC-3′ centered at −64 of the *hsp70* promoter reduced basal levels of expression approximately fivefold (Table 1 and Fig. 7; compare pΔ68 and pΔ58) (58). The complement to this sequence, GAGCCAATC, has strong homology to CAT box sequences necessary for full expression of the β-globin promoter (18, 23a. 42a). To test whether T antigen binding site 1 could function as a promoter element in a different setting, we inserted a 21-base sequence encompassing T antigen binding site 1 into the *Hin*dIII site of pΔ58, replacing the CAT box with the sequence of binding site 1 (plasmid pΔ58+1). We determined the promoter activity of this construction by cotransfecting it with pXGH5 as an internal control. This insertion resulted in a three- to fourfold activation of the promoter (Fig. 6, pΔ58+1), suggesting that this sequence can function as an upstream promoter element.

We wished to ensure that the activation of the promoter was caused by the T binding sequences and not by some artifactual juxtaposition of sequences due to insertion of the oligomer. Four sets of mutants were made that differed from plasmid pΔ58+1 only in this 21-base sequence (Fig. 6). Plasmids pΔ58+2 and pΔ58+3 both contained double point mutations that resulted in transversions of bases in the GPuGGC sequence, known to be involved in T antigen binding. Plasmid pΔ58+4 contained mutations in both GPuGGC elements. These mutants were impaired in their

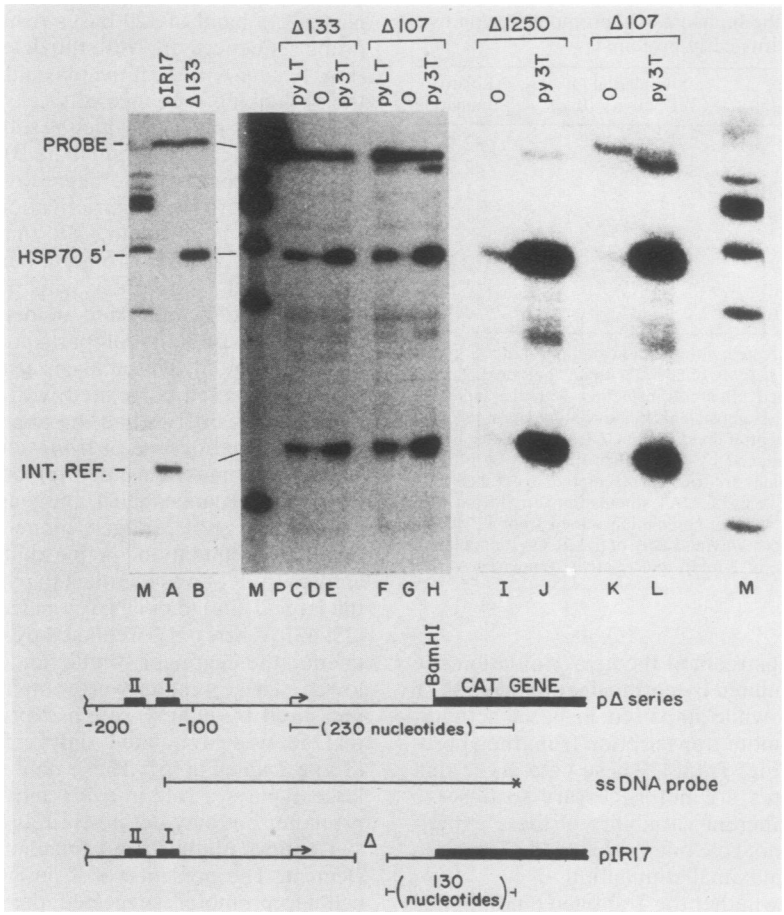

FIG. 5. Stimulation of appropriately initiated transcripts from the human *hsp70* promoter by the polyomavirus early proteins. RNA from transfected cells was analyzed by S1 nuclease analysis, and the resultant fragments were separated on an 8% polyacrylamide–urea gel. Lanes M, End-labeled *Hae*III digested ϕX174 included as marker; lane P, probe. Cells in all lanes except A and B were transfected with pIR17 (4 μg) and the following DNA: A, pIR17 (12 μg), B, pΔ133 (12 μg and no pIR17); C, pΔ133 (8 μg) and pyLT (1 μg); D, pΔ133 (8 μg); E, pΔ133 (8 μg) and py3T (1 μg); F, pΔ107 (8 μg) and pyLT (1 μg); G, pΔ107 (8 μg); H, pΔ107 (8 μg) and py3T (1 μg); I, pΔ1250 (8 μg); J, pΔ1250 and py3T (1 μg); K, pΔ107 (8 μg); L, pΔ107 (8 μg); and py3T (1 μg). The diagram at the bottom shows the promoter regions of the plasmids used (arrow is at start site) and the expected lengths of protected fragments after S1 nuclease analysis.

ability to bind large T antigen (data not shown). Plasmid pΔ58+5 had a set of changes that made the sequence identical to that of T antigen binding site 1 of SV40, a binding site that is recognized as efficiently by polyomavirus large T antigen as *hsp70* site 1 (data not shown). All four mutants were significantly reduced in activity in BALB/c 3T3 cells compared with plasmid pΔ58+1 (Fig. 6). The mutations of plasmids pΔ58+2 and pΔ58+3 both caused a twofold decrease in activity. The mutations in plasmids pΔ58+4 and pΔ58+5 both reduced activity to that of the starting plasmid, pΔ58.

Results similar to those obtained with CAT expression were obtained by analyzing RNA level (Fig. 7). Plasmid pIR17 was used as an internal control in these studies. The level of correctly initiated *hsp70* transcript from plasmid pΔ58+1 was significantly higher than that from pΔ58, implying that *hsp70* site 1 is indeed capable of functionally replacing the *hsp70* CAT box. The artificial promoter of pΔ58+1 was only minimally weaker than the wild-type *hsp70* promoter (Fig. 7, compare lanes D and E). The data imply that the sequences responsible for activity of this artificial promoter overlap with those that are responsible for binding polyomavirus large T antigen. As there was no T antigen present in these experiments, the data suggest that a cellular protein has an overlapping but not identical binding specificity as that of polyomavirus large T antigen.

DISCUSSION

Role of T antigen binding in transcriptional stimulation. Two sets of observations led to the hypothesis that the large T antigens of the papovaviruses SV40 and polyomavirus may directly interact with cellular promoters. First, viral infection results in rapid changes in the spectrum of newly synthesized cellular proteins (40). Second, these antigens in certain circumstances can confer an indefinite growth, or immortal, phenotype on primary cells which clearly entails changes in the pattern of expression of the cellular genome (45, 48). Both antigens are localized primarily in the nucleus of cells. It has been reported that SV40 large T antigen mutants that are not transported to the nucleus do not immortalize primary baby rat kidney cells (38). Although there are numerous roles that a protein can play in the nucleus to confer these phenotypes, one attractive hypothesis has been that these nuclear antigens directly modulate the activity of cellular promoters.

We have shown that polyomavirus large T antigen binds to two sites between 110 and 170 bases 5′ of the transcription

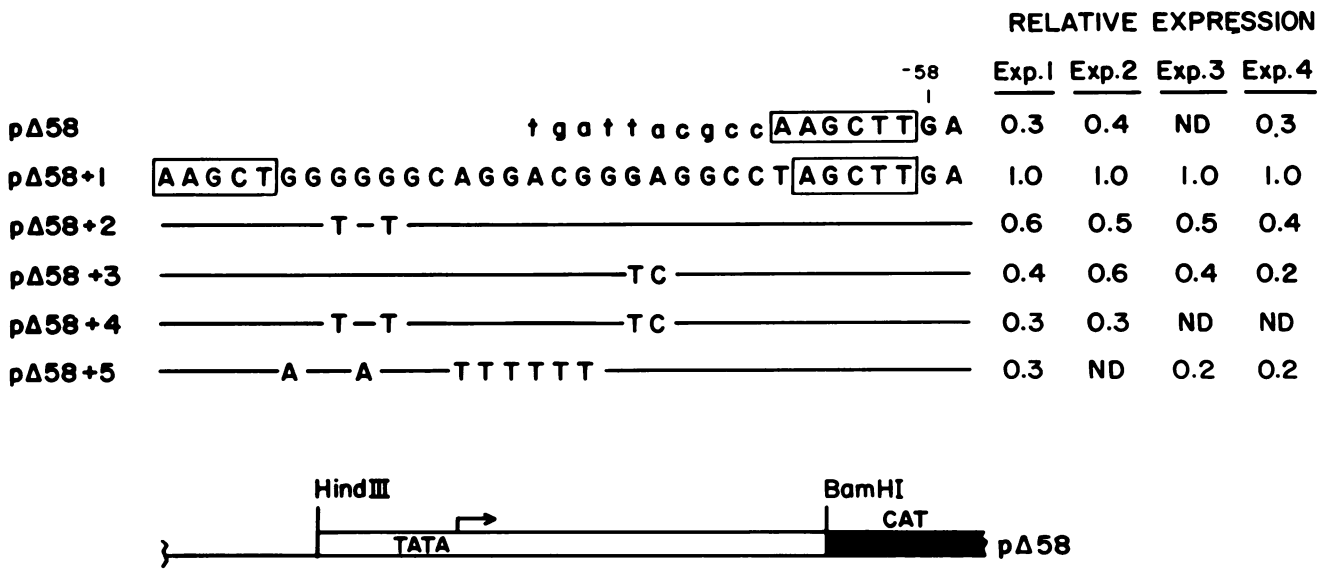

FIG. 6. Construction and mutational analysis of an artificial promoter in which human *hsp70* polyomavirus large T antigen binding site 1 functions as an upstream element. The DNA sequences surrounding the *Hin*dIII site (boxed) of plasmid pΔ58 and derivatives are shown. The solid line indicates homology with pΔ58+1. For each experiment, the indicated pΔ58 series plasmid (8 μg) was transfected with plasmid pXGH5 (2 μg). The value for relative expression was determined by correcting the level of CAT activity from the pΔ58 series plasmids for transfection efficiency as determined by growth hormone activity from pXGH5. In each experiment, pΔ58 activity was arbitrarily assigned a value of 1.0. ND, Not done.

initiation site of one of the human *hsp70* genes. These binding sites have the canonical large T antigen binding sequence defined by studies on binding to the viral genome: two pentanucleotides of the sequence GPuGGC separated by approximately one turn of the DNA double helix (12). We showed by methylation interference studies that large T antigen interacts with the predicted G residues in or adjacent to these pentanucleotide sequences in *hsp70* binding site 1. All of these G residues lie on one face of the DNA helix and contact the protein in the major groove, observations consistent with what is known for both SV40 and polyomavirus large T antigen binding to viral regulatory sequences (12, 29).

A difficulty in interpreting binding studies done with purified protein and purified DNA fragments lies in determining the biological relevance of the observed binding. The strength of the binding interaction to *hsp70* site 1 is roughly equivalent to the strength of individual large T antigen binding sites in the polyomavirus genome (Fig. 4). This

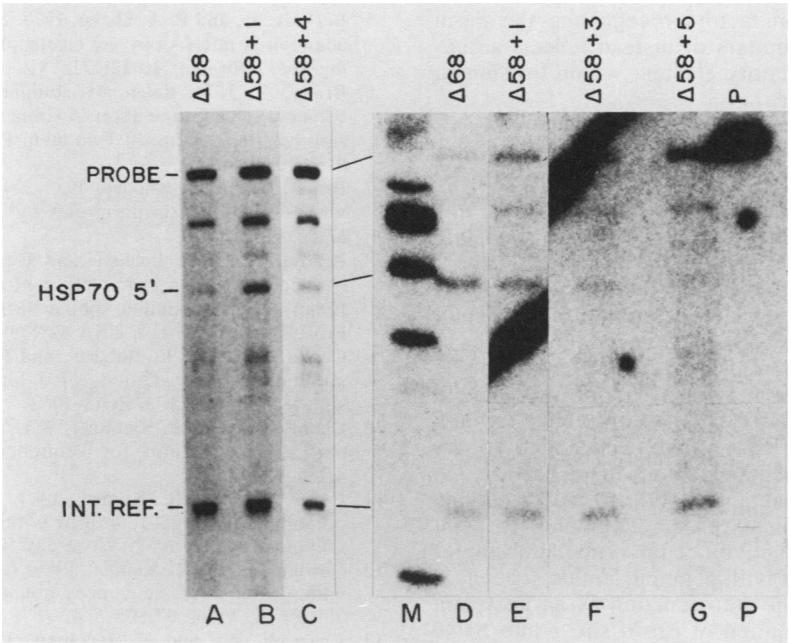

FIG. 7. Amount and structure of RNA transcribed from plasmids containing *hsp70* site I as an upstream promoter element. RNA was analyzed by S1 nuclease analysis as described in the legend to Fig. 5. Cells in lanes A through G were transfected with pIR17 (4 μg) and the following plasmid (8 μg): A, pΔ58; B, pΔ58+1; C, pΔ58+4; D, pΔ68; E, pΔ58+1; F, pΔ58+3, G, pΔ58+5. Lane M (marker) and P (probe) are as in Fig. 5. INT. REF., Transcript from internal reference plasmid pIR17.

suggests that T antigen will recognize *hsp70* site 1 in vivo, as it is capable of finding specific sites in its own genome in vivo. Does binding to *hsp70* site 1 elicit any effect? Because human cells are nonpermissive for infection by polyomavirus, we have been unable to directly assess the effect of polyomavirus infection on expression of the human *hsp70* promoter in situ. We have instead used transient transfection protocols to investigate regulation of the human *hsp70* promoter region by polyomavirus large T antigen.

Expression of the entire early region of polyomavirus results in a 5- to 20-fold stimulation of the level of correctly initiated *hsp70* transcripts (Fig. 5). However, stimulation from the *hsp70* start site is not noticeably altered by deletion of either or both of the *hsp70* T antigen binding sites. The plasmids containing these mutant promoters contained no detectable T antigen binding sites (Fig. 1E). Thus, the polyomavirus early region can activate this promoter in the apparent absence of sequence-specific binding of large T antigen. Expression from a hybrid gene containing the mouse metallothionein I promoter fused to the human growth hormone coding region was also activated by the polyomavirus early region, again suggesting that there is no strict sequence dependence for stimulation. Indirect activation of transcription has been seen with the gene products of the immortalizing regions of other DNA tumor viruses, notably the EIa region of adenovirus (24, 37, 42, 60). Previous data are consistent with the hypothesis that both the EIa proteins and SV40 large T antigen alter the function of general transcription factors in the cell (1, 7, 8, 33; reviewed in reference 36).

The data presented here argue that the polyomavirus early region proteins probably activate transcription by similar indirect means. Every tested deletion mutant of the human *hsp70* promoter that retained basal activity was stimulated by these proteins (Table 1). There was some specificity to this stimulation; the *hsp70* promoter was stimulated twice as much as the mouse metallothionein I promoter used as an internal control. Whether this specificity results from different sets of transcription factors recognizing the basal elements of these two promoters or instead reflects a contribution of a specific regulatory element within the human *hsp70* promoter is not clear.

Identification of a novel promoter element. Why are there recognition sequences for polyomavirus large T antigen in the human *hsp70* promoter? By constructing an artificial promoter in which the sequence of binding site 1 was inserted in place of the normal upstream promoter CAT box sequence, we demonstrate here that the binding site 1 sequence is capable of functioning as an upstream promoter element. Three sequence elements determine the ability of SV40 large T antigen to bind DNA; two pentanucleotides of the sequence 5′-GAGGC-3′ and a spacer sequence (15, 29, 49, 53). Polyomavirus large T antigen binding sites have a similar structure. The affinity of these proteins for any particular site appears to be partially dependent on the number of repeats of the pentanucleotide sequence (12, 49, 56). Alteration of either pentanucleotide of *hsp70* binding site 1 both eliminated polyomavirus large T binding (data not shown) and decreased the activity of the artificial promoter (Fig. 6 and 7). Alteration of both pentanucleotide sequences eliminated the ability of site 1 to function as an upstream element. Interestingly, mutation of *hsp70* site 1 into SV40 site 1, primarily by altering the constitution of the 7-base spacer sequence, also reduced the strength of the artificial promoter. This latter sequence bound polyomavirus large T antigen efficiently (data not shown) but did not appear to function as an upstream sequence. These data offer a genetic argument that a cellular transcription factor exists that has an overlapping but not identical binding specificity to that of polyomavirus large T antigen.

What role might this sequence play in regulation of the human *hsp70* promoter in vivo? It is clearly not necessary for basal activity of the promoter after transfection into established cell lines (Fig. 5). The *hsp70* promoter is regulated by numerous effects and is one of the earliest promoters turned on during embryonic development (3). An attractive hypothesis is that different upstream transcription factors play a role in regulating the promoter in the numerous settings in which it is active; the cellular factor that recognizes *hsp70* T antigen binding site 1 may be one of these factors.

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