

A single-point mutation in HCF causes temperature-sensitive cell-cycle arrest and disrupts VP16 function

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The temperature-sensitive BHK21 hamster cell line tsBN67 ceases to proliferate at the nonpermissive temperature after a lag of one to a few cell divisions, and the arrested cells display a gene expression pattern similar to that of serum-starved cells. The temperature-sensitive phenotype is reversible and results from a single missense mutation—proline to serine at position 134—in HCF, a cellular protein that, together with the viral protein VP16, activates transcription of herpes simplex virus (HSV) immediate-early genes. The tsBN67 HCF mutation also prevents VP16 activation of transcription at the nonpermissive temperature. The finding that the same point mutation in HCF disrupts both VP16 function and the cell cycle suggests that HCF plays a role in cell-cycle progression in addition to VP16-dependent transcription.

[Key Words: tsBN67; HCF protein; VP16 function; G₀/G₁ cell cycle arrest; transcription]

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Conditional mutations, particularly temperature-sensitive mutations, have been valuable tools for clarifying cell-cycle regulation in yeast as well as mammalian cells (for review, see Marcus et al. 1985). Previously, we have isolated a series of temperature-sensitive cell-proliferation mutants from the hamster BHK21 cell line (Nishimoto and Basilico 1978; Nishimoto et al. 1982). Following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, mutants that proliferate at the permissive temperature of 33.5°C but not at the nonpermissive temperature of 39.5°C were concentrated through multiple rounds of negative selection with the cytotoxic base analog 5-fluoro-2-deoxyuridine to eliminate proliferating cells at the elevated temperature. Based on the ability of hybrid cells created by the fusion of different mutant lines to grow at the nonpermissive temperature, these temperature-sensitive lines have been classified into 25 complementation groups (Nishimoto and Basilico 1978; Nishimoto et al. 1982).

To identify the genes affected by these mutations, human DNA has been used to complement the hamster-

cell proliferation defect, and the complementing human genes isolated from the rescued cells by hybridization with a human-specific DNA probe (Kai et al. 1986; Watanabe et al. 1991; Nakashima et al. 1993). Subsequently, the mutation responsible for the temperature-sensitive growth-arrest phenotype has been revealed by comparing the wild-type and temperature-sensitive cDNA sequence of the homologous hamster gene (Uchida et al. 1990; Nakashima et al. 1993; Watanabe et al. 1993).

One of our temperature-sensitive BHK21 mutants, called tsBN67, is the sole member of a complementation group displaying unique properties (Nishimoto and Basilico 1978). First, unlike other isolates, tsBN67 cells can proliferate normally for 1–2 days at the nonpermissive temperature. Second, once arrested at the nonpermissive temperature, the cells displayed high viability and could be rescued efficiently by transfer to the permissive temperature. The nature of the defect in tsBN67 cells is not known, however.

During herpes simplex virus (HSV) infection, viral immediate-early (IE) gene expression is activated by a complex of regulatory proteins that includes two cellular proteins, HCF (also termed C1, VCAF, and CFF) and Oct-1, and the viral transactivator VP16 (for review, see Thompson and McKnight 1992; O'Hare 1993). VP16 (also known as Vmw65, α TIF, and ICP25) is a virion protein that is released into the cell upon infection. After release, it associates with HCF to form a heteromeric

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complex that binds to the transcription factor Oct-1 on the *cis*-regulatory target of VP16 activation, the TAAT-GARAT motif (Gerster and Roeder 1988; Kristie et al. 1989; Katan et al. 1990; Kristie and Sharp 1990; Xiao and Capone 1990; Stern and Herr 1991).

Unlike Oct-1, a known transcription factor (for review, see Herr 1992), the cellular role of HCF is unknown. Mature HCF is a nuclear protein that is expressed broadly in proliferating cells (Kristie et al. 1995; Wilson et al. 1995a) and consists of a complex of associated polypeptides (Kristie and Sharp 1993; Wilson et al. 1993a), that represent amino- and carboxy-terminal fragments derived by highly specific proteolytic processing of a large 300-kD precursor protein (Wilson et al. 1993a, 1995b; Kristie et al. 1995). Although the function of HCF in cells is unknown, it is likely to be important because HCF activity is conserved among vertebrate and invertebrate organisms (Kristie et al. 1989; Wilson et al. 1993b).

We show here that a single missense mutation in HCF is responsible for the tsBN67 defect. At the nonpermissive temperature, tsBN67 cells resemble cells arrested in G₀/G₁ and fail to support transcriptional activation by VP16. These results indicate that HCF is important for maintenance of cellular as well as viral proliferation.

Results

Figure 1 shows a comparison of the growth rates of the parental BHK21 and temperature-sensitive tsBN67 cells at the permissive and nonpermissive temperatures. At 33.5°C, the permissive temperature, both cell lines grew at a similar rate, doubling every 18–20 hr (solid symbols). At 39.5°C, the nonpermissive temperature, however, the growth kinetics of the two cell lines differed from one another (open symbols). The BHK21 cells proliferated more rapidly at 39.5°C than at 33.5°C, doubling about every 12 hr. In contrast, although for the first 36 hr at 39.5°C the tsBN67 cells proliferated as they had at 33.5°C, after 36 hr they ceased to proliferate. Once arrested, the overall cell number remained relatively constant. These cells survived for extended periods at the elevated temperature; for example, 92% and 70% of tsBN67 cells maintained at 39.5°C for 2 and 4 days, respectively, produced colonies when transferred to 33.5°C (data not shown; see also Nishimoto and Basilico 1978). Thus, the temperature-sensitive tsBN67 cell-arrest phenotype is delayed by one to a few cell doublings and is reversible.

The HCF gene complements the tsBN67 phenotype

To identify the mutated gene responsible for the tsBN67 phenotype, high molecular weight human HeLa-cell DNA was transfected into tsBN67 cells along with the neomycin-resistant (*neo*^r) vector pSV2neo as described previously (Watanabe et al. 1991). As a negative control, tsBN67 genomic DNA was cotransfected with pSV2neo. Transformants were selected in the presence of G418 either at 33.5°C, or at 39.5°C. The tsBN67- and HeLa-

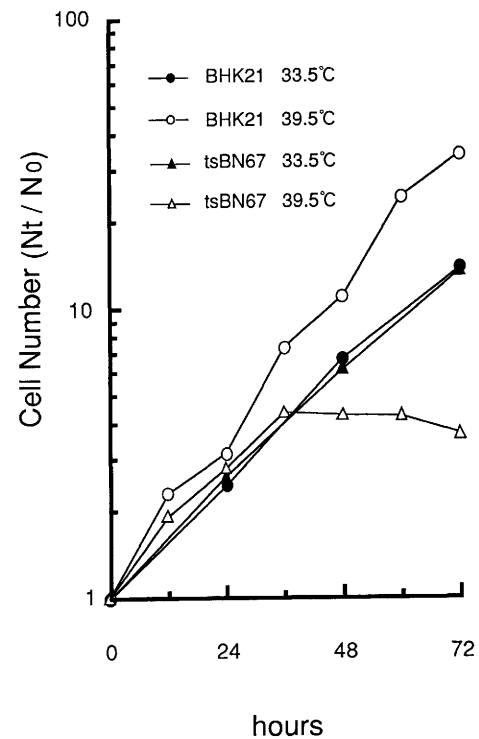


Figure 1. Hamster tsBN67 cells cease proliferating at 39.5°C after a lag of ~36 hrs. Wild-type (●,○) and tsBN67 (▲,△) BHK21 cells were seeded at densities of 1×10^5 /150-mm dish and 4×10^5 /150-mm dish, respectively, and incubated at 33.5°C for 2 days. After refeeding, half of the cultures were shifted to 39.5°C. At the indicated times, cells were harvested and counted. The ratio of cell number at each time point relative to the initial number at the temperature shift is plotted against the time of incubation. (○,△) 39.5°C; (●,▲) 33.5°C. The total cell number is a combination of floating and adherent cells.

cell DNAs displayed the same transformation efficiency at 33.5°C (Table 1). However, at 39.5°C the tsBN67 DNA failed to rescue the tsBN67 phenotype, whereas the HeLa-cell DNA yielded several ts⁺ colonies (primary transformants). Total cellular DNA prepared from one of the primary transformants, designated BN67-V-3, was transfected into the original tsBN67 cells, to enrich for human DNA essential for complementing the tsBN67 mutation.

Using a λ phage vector, we prepared a library of genomic DNA from the secondary ts⁺ transformant BN67-V-3-2, and screened for clones containing human DNA by hybridization with the human-specific *Alu* repeat probe. Three overlapping genomic DNA clones were isolated, spanning ~35 kb of human sequence (data not shown). No ts⁺ transformants were obtained upon transfection of any one of the individual clones. However, when clones 5/ λ DashII and 8/ λ DashII were transfected together, ts⁺ colonies appeared and at a higher efficiency than with total cellular DNA (Table 1; cf. experiments 1 and 2). These results indicated that the tsBN67-cell defect was complemented by either a single open reading frame (ORF) that was reconstructed by recombination of co-

Table 1. Rescue of the mutant *tsBN67* phenotype by DNA transfection

Donor DNA (+pSV2neo)	Number of colonies per 2×10^5 <i>tsBN67</i> cells	
	<i>ts</i> ⁺ , <i>neo</i> ^r	<i>neo</i> ^r
Experiment 1		
<i>tsBN67</i>	0.00	452
HeLa	0.25	452
BN67-V-3 (1st)	0.60	443
BN67-V-3-2 (2nd)	1.20	471
Experiment 2		
5/ λ DashII	0.00	160
8/ λ DashII	0.00	164
5/ λ DashII + 8/ λ DashII	13.00	164
Experiment 3		
c23/pcDL-SR α (+)	29.00	158
c23/pcDL-SR α (-)	0.00	175

Donor DNAs were transfected into *tsBN67* cells along with pSV2neo as described in Materials and Methods. The sources of DNA were as follows: (Experiment 1) High molecular weight chromosomal DNA prepared from HeLa and *tsBN67* cells, and from the primary (1st) and the secondary (2nd) *ts*⁺ *tsBN67* transformants; (experiment 2) λ phage clones containing human genomic DNA derived from the secondary (2nd) *ts*⁺ cells; (experiment 3), hamster cDNA clones inserted into the mammalian expression vector, pcDL-SR α in either the sense (+) or antisense (-) orientation. Transformants were selected in the presence of G418 (800 μ g/ml) at 33.5°C (*neo*^r) or at 39.5°C (*ts*⁺, *neo*^r) as described in Materials and Methods.

transfected genomic DNA in vivo or by two ORFs, one on each λ phage genomic DNA fragment. The results described below indicate that the complementing ORF was reconstructed by recombination after transfection.

An *Alu*-free DNA probe from clone 5/ λ DashII recognized an ~8.3-kb transcript common to both HeLa and BHK21 cells (data not shown). This probe was used to screen a hamster BHK21 cDNA library (Seki et al. 1992), and a series of clones spanning a total of 8.3 kb (and contained in the two λ phage clones 5/ λ DashII and 8/ λ DashII, described above) was obtained. Sequence analysis revealed that these cDNAs encoded the hamster homolog of the human *HCF* gene (Wilson et al. 1993a).

To confirm that expression of the single HCF ORF was responsible for rescue of the *tsBN67* mutation defect, a cDNA fragment (c23) containing a complete 6.3-kb ORF was subcloned into the mammalian expression vector pcDL-SR α in both the sense [c23/pcDL-SR α (+)] and the antisense [c23/pcDL-SR α (-)] orientation. When transfected into *tsBN67* cells, only the sense orientation construct converted *tsBN67* cells to the *ts*⁺ phenotype (see Table 1, experiment 3). This experiment demonstrates that the expression of wild-type hamster HCF cDNA alone is sufficient to complement the *tsBN67* mutation.

HCF is highly conserved between hamsters and humans

Figure 2A shows an alignment of the deduced hamster and human HCF amino acid sequences, and Figure 2B

shows a diagram of the hamster HCF ORF. The longest original human HCF ORF encoded 2035 amino acids (Wilson et al. 1993a). Some of the hamster cDNAs, however, revealed a new mRNA splicing variant that extends one exon, inserting 43 codons after codon 1508 of the hamster HCF ORF. Analysis of the original collection of human HCF cDNAs (Wilson et al. 1993a) identified a corresponding 44-codon extension in two of nine cDNA clones spanning this region of the HCF ORF (after human HCF codon 1499; data not shown); this sequence extension, arising through alternate splice acceptor site selection, has also been reported by Frattini et al. (1996). The extended hamster and human HCF sequences are included in Figure 2.

The human HCF ORF can be broadly divided into three regions: a central region containing six near-perfect 26-amino-acid HCF repeats responsible for HCF processing (Wilson et al. 1995b), and amino- and carboxy-terminal regions that remain associated with one another after HCF cleavage (Wilson et al. 1993a). The amino-terminal regions (residues 1–1009 in both hamster and human HCF) and carboxy-terminal regions (residues 1448–2090 in hamster HCF and 1439–2079 in human HCF) of hamster and human HCF are highly conserved: The amino-terminal sequences are >98% identical (15 differences in 1009 positions), and the carboxy-terminal sequences are 95% identical (34 differences in 643 positions).

In the central HCF repeat region, there is only one difference (in HCF repeat 3) among the six near-perfect HCF repeats. This high level of conservation is consistent with the strict sequence requirements for efficient proteolytic cleavage (Wilson et al. 1995b). The intervening sequences, which are frequently absent in mature HCF, are relatively hypervariable, however, displaying only 67% identity. Indeed, the hamster sequence reveals a third imperfect HCF repeat (designated HCF repeat 4B). By analogy to the imperfect and inactive human HCF repeat 7 (Wilson et al. 1995b), the imperfect hamster HCF repeat 4B is probably not active in HCF processing. The lack of high sequence conservation in the regions between HCF repeats suggests that these sequences are not critical for HCF function during or after processing.

The tsBN67 HCF gene contains a single-point mutation, which is responsible for the temperature-sensitive phenotype

To determine whether *tsBN67* cells contain a mutation in the hamster HCF gene, we isolated a series of HCF cDNA fragments that span the entire *tsBN67* HCF ORF by RT-PCR. These HCF fragments were sequenced and compared to the parental BHK21-HCF sequence. Of the 6270-base hamster HCF ORF, only a single-base substitution was identified: a C \rightarrow T transition at the first position of codon 134 (indicated by the arrow in Fig. 2A). The nucleotide sequence of this and the flanking codons is shown in Figure 2B, together with an illustration of landmarks in the HCF ORF (Wilson et al. 1993a). This single C \rightarrow T transition mutation, which is consistent with the *tsBN67*-cell mutagenesis protocol using

A

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MASAVSPANLPAVLLQPRWKRKRVVWGSFVPRPRHGRAVAIKELIVVFGGNEGIVDELHVYNTATNQWFI PAVVRGDIIPGCAAYGFVCDGTRLLVFGGM 100
VEYGYKSYNDLYELQASRWKRLKAKTPKNGPPPCCRLGHFSVLVGNKCYLFGGLANSEDPKNNIPRYLNDLYILELRPGSGVVAWDIPITYGVLPPPR 200
ESHTAVVYTEKDNKSKSLVIYGGMSGCRLGDLWTLDIETLTWNKPSLSGVAPLPRSLHSATTIGNKMYVFGGWVPLVMDVKVATHEKEWKCTNTLACLN 300
LDTMAWETILMDTLEDNIPRARAGHCAVAINTRLYIWSGRDGYRKAWNNQVCKDLWYLETEKPPPPARVQLVRANTNSLEVSWGAVATADSYLLQLQKY 400
DIPATAATATSPTPNVPSVPANPPKSPAPAAAAPAVQPLTQVGITLVPQAAAAPPSTTTIQVLPVPGSSISVPTAARAQGVPAVLKVTGPQATTGTPL 500
VTMRPAGQAGKAPVTVTSLPASVRMVVPTQSAQGTVIGSNPQMSGMAALAAAAAATQKIPSSAPTLSVSPAGTTIVKTVAVTPGTTLPATVKVASSPV 600
MVSNPATRMLKATAAQVGTSSVSAANTSTRPIITVHKSGTVTVAQQAVVTVVGGVTKTITLVKSPISVPGGSALISNLGKVMVSVQTKPVQTSVAVTQ 700
ASTGPFVTQIIQTKGPLPAGTILKLVTSADGKPTTIIITTTQASGAGSKPTILGISSVSPSTTKPGTTTIIKTIIPMSAIITQAGATGVTSTPGIKSPITIIIT 800
TKVMTSGTGAPAKIITAVPKIATGHGQQGVTVVVLKGAQPQGAILLRTVPMMSGVRLVTPVTVSAVKPAVTTLVVKGTTGVTTLGTVTGTVSTSLAGAGAH 900
STSASLATPITTLGTIATLSSQVINPTAITVSAQAQTLTAAGGLTPTTITMGPVVSQPTQVTLITAPSGVEAQPVHDLFVLSILASPTTEQPTATVTIADSG 1000
QGDVQPGVTLVLCVSNPPCETHETGTTNTATTITVAVNLGGHPQPTQVQVFCDRQEAASLVTSAVQQNGNVVRCVSNPPCETHETGTTNTATTATSNMAG 1100
QHGCNPPCETHETGTTSTATTAMSSMGTOQRDRHT--SSNPTVVRITVAPGALERTQGTVKPQCQTQQANMTNTMTVQATRSFPCPAGLLRPSVAL 1198
V ANH A RACAAGT A I S T AA S- S R TSA S M GA S G M R
EAGNHSFAFVQLALPSVRVGLSGPSNKDMPGHELETYHTYTTNTPTTALSIMGAGELGTARLIPSTYESLQASSPSSMTMTALEALLCPSATVTQVC 1298
P GR PL SK R S I L A R----- SHAVS AAMTR SV ---P MA VC-- GG T V V
SNPPCETHETGTTNTATTSNAGSAQRVCSNPPCETHETGTTHTATTATSNAGGAGQPEGGQQPAGGRPCETHQTTSTGTMSVSVGALLPDATPSHGTL 1398
T PA S R V
GLEVVAVSTVTSQAGATLLASFPTRVCSNPPCETHETGTTHTATTVTSNMSSNQDPPPAASDQGEVVSQGD SANITSSSGITTTVSSSTLPRAVTIVTQ 1498
A APS P TA P
Alternative splice (1509-1551)
STPVPGPSVNISSLTET-PGALTSEVPIPATITVTIANETSDMPFSAVDLILQPEELQVSPGPRQQLPRLQLQSASTPLMGESSEVLSASQTPPELQA 1597
K M A R T K A A
AVDLSSTGDPSSGQEPSTSSAVVAVTVVQPPPTQSEVDQLSLPQELMAEAQAGTTTLMVTGLTPEELAVTAAAEAAAQAAATEEAQALAIQAVLQAAQQA 1697
E SAG
VMAGTGPEMDTSEAAAAVTQAEGLHLSAEGQEGQATTIPVLTQOELAAALVQQQQQLQEVQAAQQQHHLPTEALAPADSLNDPSIESNCLNELASAVPS 1797
- T A H A
TVALLPSTATESLAPSNFTVAPQPVVVASPAKMQAAATLLEVDNGIESLGVKPDLP PPPSKAPVKKENQWFDVGVIKGTSVMVTHYFLPPDDAVQSDDDS 1897
L A M N P L
GTIPDYNQLKKQELQPGTAYKFRVAGINACGRGPFSEISAFKTC L PGFPGAPCAIKISKSPDGAHLTWEPPSVTSGKII EYSVYLAIQSSQAGGEPKSS 1997
V L
PAQLAFMRVYCGPSPSCLVQSSLSNAHIDYTKPAIFRIARNEKGYGPATQVRWLQETSKDSSGTPKPAKRPMSSPEMKSPAKKSKADGQ 2090
N

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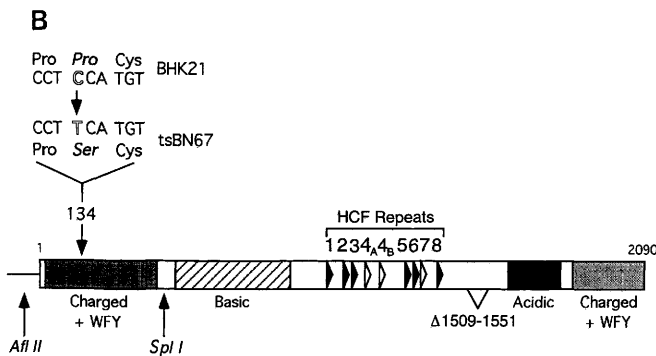


Figure 2. The hamster HCF gene is highly related to the human homolog and the mutant tsBN67 HCF gene contains a single missense mutation at position 134. (A) Comparison of the predicted hamster and human HCF amino acid sequences. The predicted sequence of wild-type BHK21 hamster HCF is shown, with residues that differ in human HCF (Wilson et al. 1993a) indicated below. The hamster sequence includes an additional 43 codons (codons 1509–1551); the corresponding human sequence (Frattini et al. 1996; this work) is included in the comparison. Heavy overlines indicate the HCF repeats; the light overline indicates the additional sequences owing to the alternative splice. (↓) Amino acid 134, which is mutated in tsBN67 cells, is indicated. The nucleotide sequence of hamster HCF has been deposited in GenBank, accession number

D45419. (B) Location of the tsBN67 mutation in hamster HCF. The general organization of the hamster HCF ORF is shown. The single nucleotide change leading to a substitution of serine for proline at position 134 is indicated. To demonstrate that this single point mutation prevents complementation by the tsBN67 HCF cDNA, an *AflII*–*SphI* fragment spanning this mutation, was exchanged for the wild-type HCF cDNA.

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N-methyl-*N'*-nitro-*N*-nitrosoguanidine [Ito et al. 1994], converts the proline at position 134 to serine.

To confirm that we had identified the relevant mutation, a 1.1-kb wild-type *AflIII-SpII* fragment, encompassing codon 134, was replaced by the corresponding tsBN67 fragment to create HCF-m14. From HCF-m14, we recreated the wild-type sequence in HCF-r14 by replacing the tsBN67 *AflIII-SpII* fragment with the corresponding wild-type fragment. Both plasmids were transfected separately into tsBN67 cells along with the pSV2neo marker and scored for colony formation at 33.5°C and 39.5°C in the presence of G418. The results of this experiment are shown in Table 2. At 33.5°C, there were similar numbers of neo^r colonies in the two transfections, indicating comparable transfection efficiencies. In contrast, at 39.5°C, colonies were identified only in the cells transfected with the HCF-r14 cDNA containing the wild-type BHK21-derived *AflIII-SpII* fragment, demonstrating that the single codon 134 point mutation in HCF-m14 is sufficient to prevent complementation of the temperature-sensitive tsBN67 phenotype.

HCF expression in tsBN67 cells is normal at the nonpermissive temperature

The HCF protein is expressed as a 300-kD precursor (HCF₃₀₀) that is processed by proteolytic cleavage into a series of amino-terminal (HCF_N) and carboxy-terminal (HCF_C) fragments [Wilson et al. 1993a]. Despite cleavage, the majority of these fragments remain tightly but noncovalently associated [Wilson et al. 1993a, 1995b]. To determine whether changes in HCF protein stability, processing, or HCF fragment coassociation in tsBN67 cells at the nonpermissive temperature might be responsible for the temperature-sensitive phenotype, we performed sequential immunoprecipitation and immunoblot analyses using HCF_N and HCF_C fragment-specific antibodies.

As shown in Figure 3, we compared HCF expression in wild-type BHK21 and tsBN67 cells incubated for 20 hr at

Table 2. The tsBN67 P134S mutation prevents complementation of tsBN67 cells by hamster HCF

Donor DNA (+pSV2neo)	Number of colonies per 2 × 10 ⁵ tsBN67 cells	
	ts ⁺ , neo ^r	neo ^r
HCF-m14	0	236
HCF-r14	25	228

The wild-type 1.1 kb *AflIII-SpII* HCF cDNA fragment in c23/pcDL-SRα(+) was exchanged for the corresponding tsBN67 fragment, introducing the tsBN67 mutation into the wild-type BHK21 HCF cDNA (HCF-m14). Afterward, the *AflIII-SpII* fragment of the HCF-m14 cDNA was exchanged with that of wild-type BHK21 HCF cDNA, reverting the mutated cDNA to the wild-type HCF sequence (HCF-r14). Both HCF-m14 and HCF-r14 recombinant cDNAs were transfected separately into tsBN67 cells, along with pSV2neo as described in Materials and Methods. Transformants were selected in the presence of G418 (800 μg/ml) at 33.5°C (neo^r) and at 39.5°C (ts⁺, neo^r).

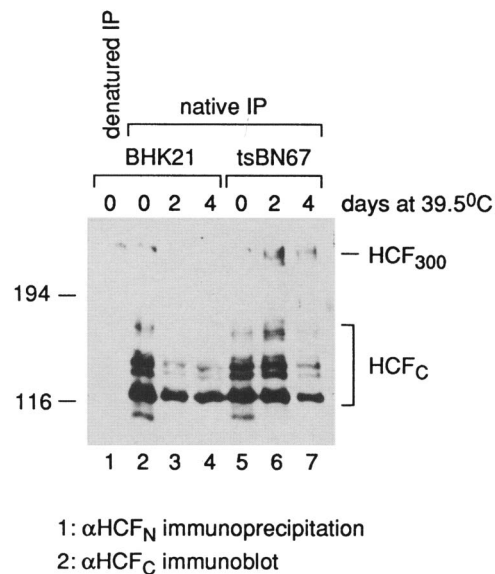


Figure 3. The tsBN67 mutation does not alter the stability or coassociation of processed HCF fragments at the nonpermissive temperature. Equivalent numbers of BHK21 (lanes 1–4) and tsBN67 (lanes 5–7) cells were plated at 33.5°C and incubated for 20 hr before being shifted to 39.5°C. Whole-cell nuclear extracts were prepared immediately (lanes 1,2,5), or after 2 (lanes 3,6) or 4 (lanes 4,7) days incubation at 39.5°C. HCF polypeptides derived from equivalent numbers of cells were recovered by immunoprecipitation with the amino-terminal HCF-specific αHCF_{N18} antiserum [see Materials and Methods]. The sample in lane 1 was heat denatured prior to immunoprecipitation. The immunoprecipitates were fractionated on a 7% SDS-polyacrylamide gel, transferred to nitrocellulose filters, and probed with αHCF_C a rabbit polyclonal antisera directed against the carboxy-terminal region of HCF (αHCF in Wilson et al. 1993a). Molecular mass markers are given in kD. The relatively low level of HCF polypeptides in lane 3 was not reproducible.

33.5°C and also subsequently for 2 or 4 days at 39.5°C. HCF polypeptides were immunoprecipitated from cell extracts with an antipeptide antiserum directed against the very amino terminus of HCF (αHCF_N). The immunoprecipitates were then fractionated by polyacrylamide gel electrophoresis and probed by immunoblot with an antibody specific for the carboxy-terminal region of HCF (αHCF_C). In the sample shown in lane 1, the BHK21 cell extract was denatured prior to immunoprecipitation; the appearance of the full-length HCF₃₀₀ polypeptide but not the processed HCF_C fragments demonstrates that under these assay conditions, recovery of the HCF_C fragments is dependent on noncovalent association with HCF_N fragments.

The pattern of wild-type hamster HCF_C polypeptides (lanes 2–4) is similar to the pattern of HCF_C polypeptides observed with human HCF [Wilson et al. 1993a], indicating that hamster HCF processing is similar to that of human HCF. Although there are temperature-induced differences in the levels of HCF_C fragments, the fragment patterns generated by both wild-type and tsBN67 cell extracts are similar (cf. lanes 2–4 and 5–7). Because

the α HCF_N epitope lies amino-terminal of the tsBN67 mutation, and the HCF_N region responsible for association with the HCF_C fragments lies carboxy-terminal of the tsBN67 mutation (Wilson et al. 1995b), recovery of HCF_C fragments with the α HCF_N antiserum demonstrates that the site of the tsBN67 mutation is intact. These results suggest that the tsBN67 mutation does not affect HCF protein stability, processing, and fragment coassociation.

VP16 function is disrupted in tsBN67 cells at the nonpermissive temperature

We next asked whether the tsBN67 HCF mutation af-

fects activation of transcription by VP16. We compared the ability of VP16 to activate transcription from a β -globin promoter containing multiple tandem VP16 response elements during transient expression in wild-type and tsBN67 BHK21 cells at the permissive and nonpermissive temperatures as shown in the RNase protection assay of Figure 4A. In the absence of VP16, the levels of β -globin transcripts were very low in both wild-type and tsBN67 cells at both temperatures (Fig. 4A, lanes 1,3,5,7). In the wild-type cells, VP16 activated this β -globin promoter at both the permissive (15-fold activation) and nonpermissive (7-fold activation) temperatures (cf. lanes 1 and 2, and 3 and 4). In tsBN67 cells, VP16 still activated

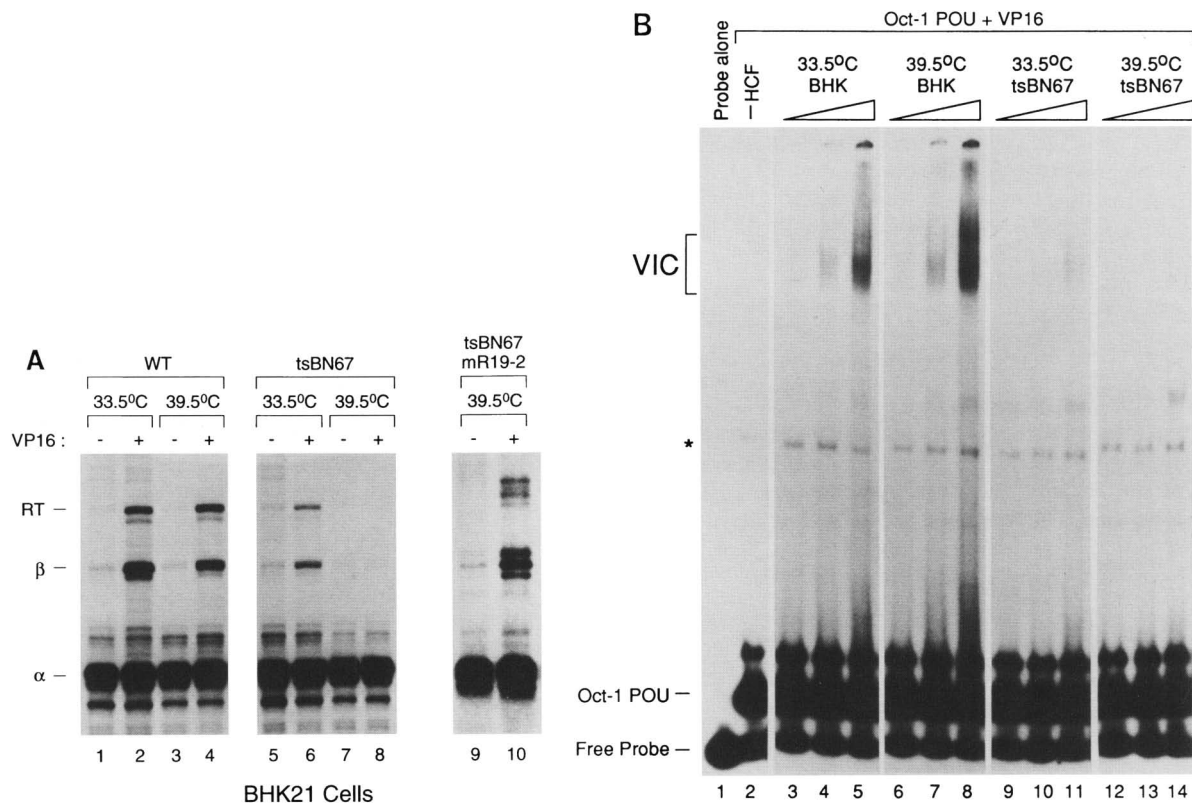


Figure 4. VP16 fails to activate transcription in tsBN67 cells at the nonpermissive temperature. (A) Transcriptional activation by wild-type VP16. VP16-dependent activation of transcription was assayed by RNase protection after transient expression in wild-type BHK21 and tsBN67 cells as described in Materials and Methods. Expression was assayed in wild-type BHK21 cells at 33.5°C (lanes 1,2) or 39.5°C (lanes 3,4), tsBN67 cells at 33.5°C (lanes 5,6) and 39.5°C (lanes 7,8), and tsBN67 mR19-2 cells (a cell line rescued by transfection of the wild-type HCF gene) at 39.5°C (lanes 9,10). All samples contained the VP16-responsive β -globin-related reporter plasmid pU2/ β 6xTAAT. The transfected cells for the samples in lanes 1,3,5,7, and 9 lacked the VP16 expression plasmid, whereas those for the samples in lanes 2,4,6,8, and 10 contained the wild-type VP16 expression plasmid. The positions of the α -globin (α), correctly initiated β -globin (β), and β -globin readthrough (RT) transcripts are indicated to the left. (B) VP16-induced complex assembly is severely reduced in extracts from tsBN67 cells. Extracts were prepared from BHK21 and tsBN67 cells maintained either at 33.5°C or 39.5°C and assayed for HCF activity in an electrophoretic mobility retardation assay, using bacterially expressed human Oct-1 POU domain, glutathione *S*-transferase VP16 (GST-VP16) fusion protein, and a labeled (OCTA⁺)TAATGARAT probe from the HSV ICP0 promoter. The addition of recombinant human Oct-1 POU domain ensures that Oct-1 is not limiting in the assay. Unbound probe (lane 1), and probe mixed with Oct-1 POU domain and GST-VP16 (lane 2) are shown. Three different amounts of each extract (1, 3, and 10 μ g) were assayed as follows: Wild-type BHK21 cells grown at 33.5°C (lanes 3–5), BHK21 cells grown at 33.5°C and shifted to 39.5°C for 48 hr (lanes 6–8), tsBN67 cells grown at 33.5°C (lanes 9–11), and tsBN67 cells grown at 33.5°C and shifted to 39.5°C for 48 hr (lanes 12–14). In this experiment, the binding reactions were performed at 37°C, but binding reactions performed at 4°C produced equivalent results (data not shown). The positions of the free probe, Oct-1 POU domain–DNA complex, and the VP16-induced complex containing hamster HCF (VIC) are indicated. The asterisk (*) indicates a weak, HCF-independent, VP16–Oct-1 POU domain complex (see lane 2).

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transcription at the permissive temperature (albeit only three-fold) but failed to activate transcription at the nonpermissive temperature (cf. lanes 5 and 6, and 7 and 8), even though general transcription was apparently not grossly disrupted because an internal reference α -globin promoter remained active. These results indicate that the single tsBN67 HCF mutation affects not only cell proliferation but also transcriptional activation by VP16. Consistent with this conclusion, in tsBN67 cells rescued for growth at the nonpermissive temperature (called mR19-2), VP16 transcriptional activation is also restored at the nonpermissive temperature (cf. lanes 9 and 10).

VP16 might fail to activate transcription in tsBN67 cells at the nonpermissive temperature because (1) formation of the VP16-induced complex is impaired, or (2) HCF in the VP16-induced complex is deficient for a function required to activate transcription. To discriminate between these two possibilities, we assayed the ability of cell extracts prepared from wild-type and tsBN67 BHK21 cells grown at 33.5°C or at 39.5°C for 48 hr to support VP16-induced complex formation in an electrophoretic mobility retardation assay, as shown in Figure 4B. The relative levels of VP16-induced complex formation (labeled VIC) were similar to the relative levels of VP16-dependent activation of transcription in Figure 4A: VP16-induced complex formation was readily detected in wild-type cells grown at either 33.5°C (lanes 3–5) or 39.5°C (lanes 6–8), but was significantly reduced in the extracts from tsBN67 cells grown at 33.5°C (lanes 9–11) and below our detection levels in tsBN67 cells grown at 39.5°C (lanes 12–14). Thus, VP16 apparently fails to activate transcription in tsBN67 cells at the nonpermissive temperature because the mutant HCF does not effectively support formation of the VP16-induced complex. Consistent with this hypothesis, VP16-induced complex formation is restored in the rescued mR19-2 tsBN67 cell line (data not shown).

tsBN67 cells arrest at the nonpermissive temperature primarily with a 2C DNA content

To characterize the cell-cycle status of arrested tsBN67 cells, we performed fluorescence-activated cell sorting (FACS) on cycling and arrested cells. Figure 5A shows a FACS analysis of parallel tsBN67 cultures incubated at either 33.5°C or 39.5°C for 48 hr. At the permissive temperature, tsBN67 cells show a typical profile for an asynchronous population of cycling BHK cells, with 42.8%, 43.2%, and 14% of the cells in G_1 , S, and G_2/M , respectively. In contrast, after incubation at the nonpermissive temperature, many fewer tsBN67 cells were in S phase (10.9%). Instead, the large majority (73.1%) had a 2C DNA content, consistent with an arrest in either G_1 or G_0 . The 4C DNA content peak (16% of cells) may represent tsBN67 cells arrested in G_2/M or alternatively may represent tetraploid G_1 cells. We have not distinguished between these possibilities. In either case, however, these results indicate that after initiation of DNA replication at the nonpermissive temperature, the majority of tsBN67 cells do not arrest until sometime after its

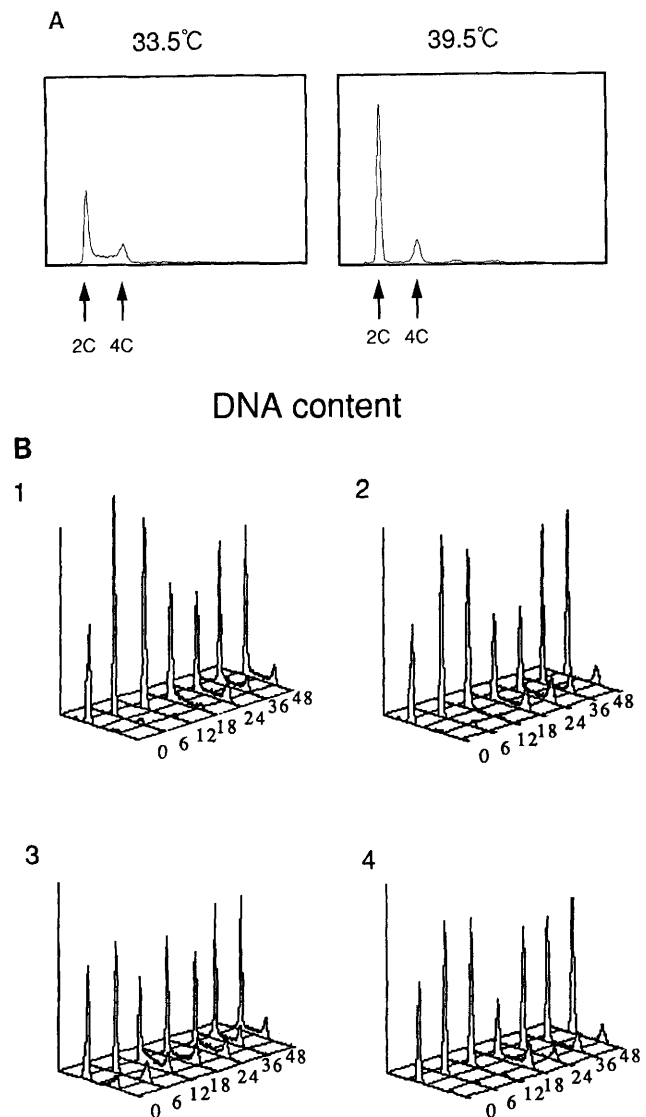


Figure 5. Cell cycle analysis of tsBN67 cells. (A) DNA content FACS analysis of growing and arrested tsBN67 cells. Exponentially growing cultures of tsBN67 cells were seeded at 1×10^6 /100-mm dish and grown at 33.5°C. After 2 days incubation, half the plates were shifted to 39.5°C, and after 48 hr, cells held at each temperature were prepared for FACS analysis as described in Materials and Methods. (B) DNA content of tsBN67 and wild-type BHK21 cells traversing the cell cycle following release from serum starvation at 39.5°C. Exponentially growing cultures of wild-type BHK21 (panel 3) and tsBN67 (panels 1,2,4) cells were incubated in DMEM medium containing 0.25% calf serum for 48 hr at 33.5°C (panels 1,2) or 18 hr at 33.5°C followed by 30 hr at 39.5°C (panels 3,4), after which the medium was exchanged for preheated complete medium containing 10% fetal calf serum, and the cells were incubated further at 33.5°C (panel 1) or 39.5°C (panels 2–4). At the indicated times following serum addition, cells were prepared for FACS analysis as described in Materials and Methods.

completion, mostly with a 2C DNA content characteristic of G_1 and G_0 cells.

The growth curves in Figure 1 show that there is a lag of a few cell divisions before tsBN67 cell-cycle arrest

occurs at the nonpermissive temperature. We imagine two possible explanations for this delay: (1) a proliferation-independent mechanism in which, for example, it simply takes time for the elevated temperature to inactivate the tsBN67 HCF protein, or (2) proliferation-dependent mechanisms in which proliferation at the nonpermissive temperature is required either to inactivate the tsBN67 HCF protein or to inactivate/deplete a hypothetical protein whose expression is regulated by HCF. To discriminate between these proliferation-independent and -dependent mechanisms, we incubated the tsBN67 cells at the permissive temperature in low serum (0.25%) for 18 hr to cause G₀ arrest and subsequently continued maintaining the arrested cells in low serum for 30 hr but at the nonpermissive temperature. If the delay is proliferation independent then incubation of the serum-arrested cells at the nonpermissive temperature should still impair subsequent cell proliferation after addition of serum, whereas if the tsBN67 phenotype is dependent on proliferation at the nonpermissive temperature then release from serum arrest at the nonpermissive temperature should result in one or more rounds of cell division.

Figure 5B (panel 4) shows a series of FACS analyses resulting from time points taken at and after addition of serum in such an experiment. At the time of serum addition, the arrested tsBN67 cells contained primarily a 2C DNA content (0-hr sample; panel 4), consistent with arrest in G₀. The refed tsBN67 cells entered S phase at the nonpermissive temperature with some delay compared with wild-type BHK21 cells (cf. 12-hr profiles; panels 3 and 4). The tsBN67 cells subsequently traversed about one round of the cell cycle before accumulating with primarily a 2C DNA content (see 48-hr tsBN67 profile in Fig. 5B, panel 4). These findings suggest that the tsBN67 cell-cycle arrest is dependent on proliferation at the nonpermissive temperature; perhaps passage through the cell cycle is required to inactivate HCF or to inactivate or deplete a critical cellular factor that is regulated by HCF. This hypothesis was confirmed further by the finding that tsBN67 cells incubated at the permissive temperature in low serum for 48 hr were arrested after about one round of the cell cycle at the nonpermissive temperature, after release from serum starvation (cf. panel 2, release from serum starvation at nonpermissive temperature, and panel 1, release from serum starvation at the permissive temperature).

Early G₁ gene expression profiles are similar in tsBN67 cells arrested by nonpermissive temperature or serum starvation

To characterize the cell-cycle status of tsBN67 cells arrested by incubation at the nonpermissive temperature, we compared the steady-state levels of *c-jun*, *fra-1*, and *c-myc* transcripts in cycling and arrested tsBN67 cells by Northern blot analysis as shown in Figure 6. These three genes represent examples of IE (*c-jun*) and delayed-early (*c-myc* and *fra-1*) genes expressed during G₁ (Lau and Nathans 1991). Asynchronous cycling cultures of

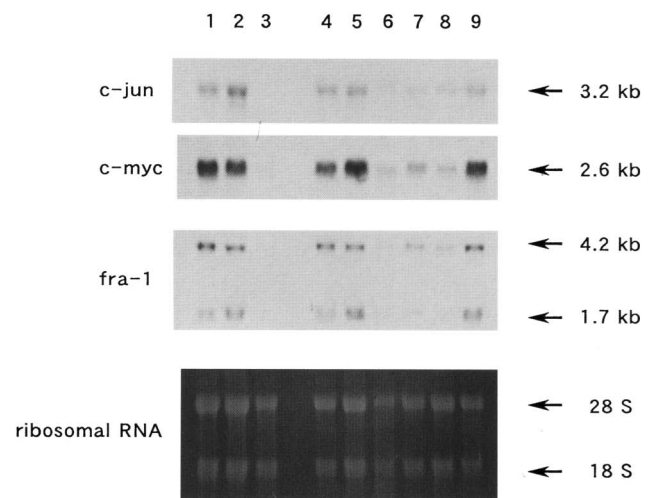


Figure 6. Northern blots of RNA prepared from growing and arrested cultures of wild-type BHK21 and tsBN67 cells probed for *c-jun*, *fra-1* and *c-myc* transcripts. Asynchronous cultures of wild-type BHK21 (lanes 1–3) and tsBN67 (lanes 4–9) cells were seeded at 33.5°C. After 2 days, one series of cultures was shifted to 39.5°C and total RNA was prepared after incubation for 0 (lanes 1,4), 12 (lane 5), 24 (lane 6), and 48 (lanes 2,7) hr. Another series of cultures was incubated for 48 hr at 33.5°C in normal medium (lane 9) or in low serum medium (lanes 3,8), and then total RNA was isolated. RNAs were probed by Northern blotting using probes derived from *c-jun*, *fra-1*, and *c-myc* cDNAs as described in Materials and Methods. The levels and integrity of the RNA in each sample were examined by staining the rRNA with ethidium bromide as shown in the *bottom* panel.

tsBN67 cells were shifted from 33.5°C to 39.5°C, and total RNA was isolated after 0, 12, 24, and 48 hr incubation (Fig. 6, lanes 4–7). As controls, asynchronous cycling cultures of wild-type BHK21 cells were incubated at 39.5°C for 48 hr (lane 2), and wild-type BHK21 and tsBN67 cells were incubated at 33.5°C in low serum medium for 48 hr to arrest cells at G₀/G₁ (lanes 3 and 8, respectively). Compared to asynchronous cycling cells (lanes 1,4), the level of *c-jun*, *c-myc*, and *fra-1* transcripts dropped significantly in both wild-type BHK21 and tsBN67 cells incubated in low serum medium at 33.5°C (lanes 3,8). At 39.5°C, no reduction of *c-jun*, *c-myc*, and *fra-1* transcripts was observed even after incubation for 48 hr in wild-type BHK21 cells (lane 2); in tsBN67 cells, however, the level of these transcripts dropped significantly after 24 hr incubation (cf. lanes 4 and 5 to lanes 6 and 7)—levels similar to those in the cultures arrested by serum starvation. The similarity in G₁-gene expression pattern between the temperature- and serum-arrested tsBN67 cells suggests that tsBN67 cells arrested at 39.5°C enter a G₀-like state.

Discussion

We have identified the gene and mutation responsible for the temperature-sensitive growth-arrest phenotype of the hamster tsBN67 BHK cell line. The mutation is a

single amino acid substitution—proline to serine— at position 134 of HCF. These results demonstrate that in addition to a role in HSV infection, HCF has a role in cell proliferation. Remarkably, at the nonpermissive temperature, the single tsBN67 amino acid substitution interferes with the role of HCF in both cell cycle progression and activation of transcription by VP16, suggesting that parallels exist in how HCF promotes these two processes. These insights into the role of HCF in cell proliferation were made possible by the conditional phenotype of the tsBN67 mutation.

HCF may function as a determinant for entry into G₀

The phenotype of the cell-cycle-arrested tsBN67 cells displays a number of interesting properties. First, the arrest is not immediate at the nonpermissive temperature, and even if tsBN67 cells arrested by serum starvation are incubated at high temperature, they will resume cycling after addition of serum. Second, based on DNA content, the arrest is primarily in G₁ or G₀, and the early G₁ gene expression pattern of temperature-arrested cells suggests that they enter a G₀-like state. Third, arrested tsBN67 cells are stable at the nonpermissive temperature.

At first, the delay in tsBN67–cell arrest at the elevated temperature seems paradoxical given that in the original isolation protocol, cells that proliferate at elevated temperature were selected against by treatment with the cytotoxic base-analog 5-fluoro-2-deoxyuridine (Nishimoto and Basilico 1978). Perhaps the arrest of tsBN67–cell proliferation is stochastic at the elevated temperature such that some cells arrest early and, because these cells are very stable at the elevated temperature, once arrested survive the selection protocol efficiently. This possibility would explain why tsBN67 cells were only isolated after three rounds of 5-fluoro-2-deoxyuridine selection, whereas other cell lines such as tsBN2, which have a rapid cell-cycle arrest phenotype but are unstable at the elevated temperature, appeared after the first round of selection and disappeared after the second and third rounds of selection (Nishimoto and Basilico 1978).

The ability of tsBN67 cells released from arrest by serum starvation to enter S phase, irrespective of preincubation at the nonpermissive temperature (Fig. 5B), suggests that HCF acts prior to the serum-arrest point of the cell cycle. This conclusion is consistent with the finding that the expression pattern of early G₁ genes in tsBN67 cells arrested at the nonpermissive temperature is similar to that of tsBN67 cells arrested by serum starvation (Fig. 6). Thus, tsBN67 cells apparently arrest in a G₀-like state at the nonpermissive temperature. Perhaps, HCF is required for promoting passage from G₀ to G₁. The G₀-like properties of arrested tsBN67 cells may be the reason for their high survival rate at the nonpermissive temperature because G₀ cells are known to survive for long periods of time as growth-arrested cells. A series of genes that are expressed specifically in growth-arrested cells (GAS genes) has been described (Schneider et al. 1988). Studies of their expression in temperature-arrested

tsBN67 cells should further define the nature of the tsBN67 arrest point and its relationship to G₀.

VP16 targets a cell-cycle regulator

In contrast to some temperature-sensitive cell-cycle arrest mutations that lead to instability of the mutant protein (see, e.g., Nishitani et al. 1991; Nakashima et al. 1993), the tsBN67 mutation has no apparent effect on the stability, maturation, or coassociation of processed HCF fragments. It does, however, apparently affect the ability of HCF to stabilize the VP16-induced complex because HCF-containing extracts prepared from tsBN67 cells incubated at the nonpermissive temperature fail to promote VP16-induced complex formation. This defect explains why VP16 does not activate transcription effectively in these cells at the nonpermissive temperature, which in turn presents strong evidence that HCF is important for transcriptional activation by VP16 in vivo and therefore a key regulator of HSV IE gene transcription.

The parallel effect of the tsBN67 mutation on VP16 function and cell proliferation raises the possibility that VP16 associates with HCF by mimicking a cellular protein that regulates cell proliferation. By analogy to the VP16-induced complex in HSV-infected cells, the cellular protein mimicked by VP16 may form a multiprotein complex with HCF and regulate transcription in uninfected cells. Because there is currently no evidence that Oct-1 interacts with HCF in uninfected cells, we do not hypothesize that Oct-1 is a member of such an HCF-containing regulatory complex. We do suggest, however, that such a regulatory complex explains the characteristic delay in growth arrest of tsBN67 cells at the nonpermissive temperature because continued cell proliferation may be required to deplete a cellular factor regulated by this putative HCF-containing complex that promotes cell proliferation, particularly through the G₀/G₁ decision point. The identification of a cellular protein mimicked by VP16 will be particularly interesting, because it may help clarify the molecular mechanisms by which cells decide between G₀ and G₁.

The VP16–HCF interaction: a sensor of cell status during HSV infection

Unlike obligate lytic viruses, HSV can undergo a productive lytic infection pathway or be latent for long periods. This dual course of infection is influenced by the status of the infected cell. Infection of epithelial cells often leads to productive lytic infection, whereas infection of neurons, in particular those in the trigeminal and dorsal root ganglia, leads to latent infection (for review, see Roizman and Sears 1987). By associating with two cellular factors, HCF and Oct-1, and subsequently activating transcription of HSV IE genes, VP16 may serve as a sensor of cell status that contributes to the viral decision to undergo a productive lytic infection or become latent. The function of HCF revealed by the tsBN67 mutation—that it promotes progression through G₁—suggests that

it is an excellent target for the capacity of VP16 as a cell-status sensor because cells infected by HSV are commonly in the G₀ or G₁ phases of the cell cycle.

In cultured cells, HSV grows productively in G₁ cells, promoting progression through G₁ (Hilton et al. 1995) but inhibiting entry into S phase (de Bruyn Kops and Knipe 1988). In these cells, VP16 originating from the tegument of the infecting virion can associate with HCF and promote productive infection. In animals, neurons of the trigeminal ganglion, in which HSV establishes a latent infection, are predominantly G₀ cells. In such cells, if HCF is inactive, tegument-derived VP16 may fail to promote productive infection. In latently infected neurons, VP16-HCF association may then serve as a key regulator for entry into productive viral infection by responding to the G₀/G₁ status of the infected cell. Knipe and colleagues (Kosz-Vnenchak et al. 1993) have suggested that during latent infection of trigeminal ganglion neurons, HSV DNA is replicated at low levels, resulting in low-level expression of late gene products such as VP16. Under such conditions of low-level replication and late-gene expression, subtle changes in the abundance of active HCF could influence the ability of VP16 to amplify the reactivation process and thus respond to changes in the G₀/G₁ status of the latently infected cell. The association of VP16 with HCF appears ideally suited for such a regulatory role because of the involvement of HCF in the G₁ phase as revealed by the tsBN67 mutation.

Materials and methods

Cell lines and cell culture

All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum or 10% fetal calf serum in a humidified atmosphere containing 10% CO₂. tsBN67 cells were maintained at 33.5°C, the permissive temperature, and ts⁺ cells were selected at 39.5°C, the nonpermissive temperature.

Stable transformantion of tsBN67 cells

High molecular weight HeLa-cell DNA (20 µg/dish), or cloned genomic DNA or cDNA expression clones (2 µg/dish), were transfected into tsBN67 cells (seeded at 2 × 10⁵ cells/100-mm dish) along with the neo^r pSV2neo expression vector (2 µg/dish), using either the calcium phosphate coprecipitation method (genomic DNA) or a modification of the Chen and Okayama method (cDNA expression plasmids), as described previously (Watanabe et al. 1991). Transfected cells were incubated at 33.5°C for 18 hr, washed with TD (Tris-buffered saline without Ca²⁺ and Mg²⁺), and, after a further 48 hr incubation at 33.5°C, cultured in the presence of G418 (800 µg/ml) at either 33.5°C or 39.5°C. The number of colonies was determined after incubation for about 2 weeks as described (Watanabe et al. 1991).

Construction and screening of DNA libraries

A library of genomic DNA clones from ts⁺ tsBN67 transformants was constructed in the λ DashII vector (Stratagene, Inc.) and screened for human sequences using a human *Alu* repeat-containing plasmid as probe. A BHK21-cell cDNA library, con-

structed in the λgt10 vector, was then screened, using an *Alu*-free region from human genomic DNA clone 5/λDashII, as described previously (Watanabe et al. 1991).

HCF immunoprecipitation and immunoblotting

A 19-amino-acid peptide with the sequence CMASAVSPANLPAVLLQPR, containing HCF residues 1–18 (underlined) was synthesized, coupled to keyhole limpet hemocyanin, and inoculated into a New Zealand white rabbit with 300 µg of antigen in Freund's incomplete adjuvant at 3-week intervals (Harlow and Lane 1988). An immune response was detected by mobility retardation analysis. HCF immunoprecipitation was performed with this αHCF_N antipeptide antiserum, referred to as αHCF_{N18}, at a 1:100 dilution. Immunoprecipitation from native and denatured extracts and subsequent immunoblotting were performed as described previously (Wilson et al. 1993a, 1995b).

Transient expression assay

Wild-type and tsBN67 BHK cells grown at 33.5°C were seeded at 1.2 × 10⁶ cells/100-mm dish for assay at 33.5°C, and 6 × 10⁵ cells/100-mm dish for assay at 39.5°C. Immediately after seeding, the cells were placed at the assay temperature (either 33.5°C or 39.5°C) and then transfected 24 hr later by calcium phosphate coprecipitation essentially as described by Cleary et al. (1993). Briefly, each plate was transfected with 4 µg of the VP16-responsive β-globin-related reporter plasmid pU2/β 6xTAAT, along with 200 ng of the internal reference plasmid pα4x(A+C), 80 ng of a wild-type VP16 expression plasmid (pC-GNVP16) where appropriate, and pUC119 carrier DNA to a total of 20 µg DNA. Cells were washed with 1 × PBS/1 mM EGTA 24 hr post-transfection and collected 12 hr later. To measure reporter and internal reference transcripts, cytoplasmic RNA was prepared by the NP-40 lysis method and probed with radiolabeled antisense β-globin (β134) and α-globin (α98) probes (Cleary et al. 1993). Unprotected RNA was digested with RNase A and T₁, and the resulting protected RNAs separated on a 6% denaturing polyacrylamide gel. Levels of reporter gene transcription were quantitated using the Fuji BAS1000 PhosphorImager and normalized to the level of the internal reference α-globin RNA.

Electrophoretic mobility retardation assays

Extract preparation and electrophoretic mobility retardation assays were performed as described (Wilson et al. 1993a), except that fetal bovine serum was not added to the binding reactions.

FACS analysis

To determine cellular DNA content, cells were removed from plates with trypsin, washed with PBS, fixed with 50% ethanol, treated with RNase A (1 mg/ml), and stained with propidium iodide (0.1% sodium citrate, propidium iodide 50 µg/ml). Cellular DNA content were then analyzed by FACScan (Becton-Dickinson).

Northern analysis

RNA filters were prepared as described previously (Watanabe et al. 1991) and prehybridized with 100 µg/ml of salmon sperm DNA at 42°C for 18–24 hr in buffer containing 0.5% SDS, 50% formamide, 6 × SSC (0.15 M NaCl, 0.015 M sodium citrate at pH 7.0), 5 × Denhardt's solution, and then incubated for 24–48 hr, with ³²P-labeled *c-jun*, *c-myc*, and *fra-1* cDNA fragments de-

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rived as follows: *c-jun* [2.6-kb *RsrII-ScaI* fragment of murine *c-jun* (Ryder and Nathans 1988)], *c-myc* [1.5-kb *SacI-HindIII* fragment of murine *c-myc* (Nakabeppu et al. 1988)], and *fra-1* [0.4-kb *KpnI-XbaI* fragment of murine *fra-1* (Cohen and Curran 1988)]. After hybridization, the filters were washed in the following manner: twice in 2× SSC plus 0.1% SDS for 30 min at room temperature, twice in 1× SSC plus 0.1% SDS for 30 min at 70°C, and then in 0.2× SSC plus 0.1% SDS at 70°C for 60 min. Finally, filters were dried and analyzed with a Fuji Bioimage analyzer.

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