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Isolation and Preliminary Characterization of the Chinese Hamster Thymidine Kinase Gene

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The Chinese hamster thymidine kinase (TK) gene has been isolated from a recombinant phage library constructed with genomic DNA from mouse Ltk^- cells transformed to Tk^+ by transfection with Chinese hamster genomic DNA. The phage library was screened by the Benton-Davis plaque hybridization technique, using as probes, subclones of recombinant phage that were isolated from mouse Ltk^+ transformants by the tRNA suppressor rescue method. The Chinese hamster TK gene is contained within 13.2 kilobases of genomic DNA in the isolate designated λ34S4. This gene, defined by restriction enzyme sensitivity experiments, homology studies with the chicken TK gene, and mRNA blotting experiments, may extend over 8.5 kilobases. Subclones of the λ34S4 isolate used as hybridization probes identified a 1,400-nucleotide polyadenylated RNA as the hamster TK mRNA. The abundance of this mRNA varies dramatically in Chinese hamster cells cultured under various growth conditions, providing direct evidence that the growth dependence of TK activity may be regulated in an important way at the level of cytoplasmic TK mRNA.

The activity of the enzyme thymidine kinase (TK) (EC 2.7.1.21) in mammalian cell cultures has been understood since the early 1960s to be dependent on cell culture growth conditions. The TK activity in asynchronously growing cell cultures, for example, is maximal through the mid-log phase but diminishes sharply as such cultures grow to stationary confluence and cells withdraw from the division cycle (7, 16, 18, 24, 30). With the development of techniques to synchronize the division cycles of cells in vitro (2, 37, 45), it was readily established that the TK activity in growing cell cultures is associated primarily with cells in the S phase of the cell division cycle. TK activity was found to be virtually undetectable in cells in the G_1 phase, to increase sharply as cellular DNA is replicated in the S phase, and then to decline as cells progress through the G_2 phase to mitosis (3, 19, 25, 27, 35, 36). TK gene expression, therefore, shows an S phase specificity (as do other enzymes) that is important to the biosynthesis of DNA nucleotide precursors, notably, dihydrofolate reductase and thymidylate synthetase (11, 15, 17, 22, 26, 28, 41, 44).

The mechanisms which govern the growth and cell-cycle-phase specificity of TK gene expression have not been precisely defined. It seems clear, however, that the periodicity of TK activity is not accomplished through a cyclic modification or inhibition of the TK enzyme (18), but depends on the appearance and translation of TK mRNA in the S phase cytoplasm (16, 19, 27). Whether such phase-specific increases in cytoplasmic TK mRNA are mediated by a transcriptional activation of the TK gene locus or by posttranscriptional processing events remains to be established. In this regard, it is important to note that recent studies of dihydrofolate reductase gene expression in cell lines with amplified dihydrofolate reductase gene loci suggest the importance of posttranscriptional events to the S phase-specific expression of the dihydrofolate reductase gene (17, 22).

In 1981, Schlosser et al. (32) generated, by transfection, mouse cell lines containing either the rat, hamster, or human TK gene and demonstrated, using synchronized cell cultures, that the expression of the transfected mammalian TK genes was S phase specific. These experiments established directly that the genetic determinants which govern the cell cycle dependence of TK gene expression can be cotransfected with, and must, therefore, be closely linked to, mammalian TK structural gene sequences. These results raise the possibility that such determinants can be localized and further defined by systematically constructing mutants of a cloned mammalian TK gene which could be analyzed

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for cell cycle-dependent expression after transfection into a genetically homologous Tk^- cell host. This report describes the isolation of the mammalian Chinese hamster TK gene, which was selected for study based on preliminary experiments which demonstrated that this gene, in DNA from the Chinese hamster ovary (CHO) A-29 cell line, could readily transform mouse Ltk^- cells, in which environment its enzyme was stable and easily assayed.

MATERIALS AND METHODS

Cell culture and DNA transfection. Mouse Ltk^- $Aprt^-$ cells (42) and A-29 CHO cells (8) were maintained in Dulbecco modified Eagle medium containing 10% bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 10 U of penicillin per ml, and 10 μg of streptomycin per ml at 37°C in a humidified atmosphere containing 5% CO_2. For DNA transfections, mouse Ltk^- cells were seeded at 5×10^5 cells per 100-mm culture dish (Nunc) and transfected 24 h later. The preparation of calcium phosphate precipitates and hypoxanthine-aminopterin-thymidine (HAT) selective regimens was as described elsewhere (42). Transfections with cloned DNAs or restriction enzyme-digested DNAs were conducted in the presence of 20 μg of high-molecular-weight Ltk^- genomic DNA per ml.

Preparation and analysis of cellular DNA and RNA. High-molecular-weight DNA for transfections and Southern blot analysis was extracted from confluent monolayer cultures in 10 mM Tris-hydrochloride (pH 7.4)–400 mM NaCl–5 mM EDTA–2% sodium dodecyl sulfate–100 μg of proteinase K per ml and extracted organically as described elsewhere (42).

The DNAs were digested with restriction enzymes under conditions recommended by the vendors (New England Biolabs, Beverly, Mass. or Bethesda Research Laboratories, Gaithersburg, Md.). Restriction digests were electrophoresed through 1.0% agarose (Sea-Kem) gels in a running buffer of 40 mM Tris-hydrocholoride (pH 7.8)–50 mM sodium acetate–1 mM EDTA at 50 V for 16 h and then transferred to nitrocellulose filters (type HA 85; Schleicher & Schuell Co., Keene, N.H.) by the method described by Southern (34). DNA probes were generated by nick-translation as described by Postel and Levine (30) and hybridized at 60°C in 6× SSC (1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate) as previously described (29).

Restriction digests of 150 μg of genomic DNA were size fractionated on 36 ml of 10 to 40% sucrose gradients prepared in a buffer of 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl–5 mM EDTA. Gradients were centrifuged in an SW27 rotor for 30 h at 24,000 rpm at 20°C and fractionated from below by needle puncture.

For the isolation of RNA from mid-log cultures of A-29 cells, the cells were plated at a density of 5×10^5 cells per 100-mm dish and harvested 3 days later without refeeding when the cells reached a density of 2×10^6 cells per 100-mm dish. RNA was isolated from confluent cultures of A-29 which were held without refeeding for 36 to 48 h after the cultures reached a saturation density of 2×10^7 cells per 100-mm dish. Cytoplasmic RNA was extracted from postnuclear supernatants by the urea-sodium dodecyl sulfate method as described elsewhere (13, 33).

Polyadenylated [poly(A)$^+$] RNA was selected from preparations of cytoplasmic RNA by oligodeoxythymidylic acid-cellulose chromatography essentially as described by Lewis et al. (21). Poly(A)$^+$ RNA was denatured at 55°C in the presence of 50% deionized formamide–2.2 M formaldehyde and fractionated by electrophoresis through 1.8% horizontal agarose gels containing 2.2 M formaldehyde in a running buffer of 20 mM morpholinepropanesulfonic acid (pH 7.0)–5 mM sodium acetate–1 mM EDTA essentially as described elsewhere (20). RNAs were transferred to nitrocellulose filters (type HA85; Schleicher & Schuell) and hybridized with nick-translated DNA probes as described elsewhere (38).

Molecular cloning. All enzymes for molecular cloning were obtained from New England Biolabs or Bethesda Research Laboratories.

All λ phage were propagated in NZCYM broth on *Escherichia coli* BNN45 (6). Phage DNA was recovered from lysates of 100-ml cultures by the glycerol step-gradient method (39) or from liter-culture lysates by the CsCl equilibrium centrifugation method (46).

The cloning arms of λ1059 *Sam7* and λL47 were prepared from phage DNA purified from CsCl-banded virions purified by the method of Yamamoto et al. (46). Restriction digests were electrophoresed through 0.7% agarose gels, and the appropriate cloning arm fragments were recovered from the gel by the KI extraction method (40).

Recombinant phage were prepared by the ligation of phage arms and genomic DNA at a mass ratio of 2:1 at 9°C for 36 h at a final concentration of 300 μg/ml. These ligates were packaged in vitro, using extracts from strains BHB2688 and BHB2690 as described elsewhere (12).

Recombinant λ1059 *Sam7* phage, which plaqued on lawns of $SupF^-$ *E. coli* KS624 (K. Shimizu, J. A. Lewis, M. Goldfarb, and M. H. Wigler, submitted for publication), were analyzed for the presence of an *E. coli* tRNA *supF* gene by spot test on lawns of KS624 prepared with isopropyl-β-D-thiogalactopyranoside and X-GAL (7).

*Eco*RI fragments from digests of recombinant phage isolates were ligated to bacterial alkaline phosphatase-treated *Eco*RI-cleaved pBR322 (Bethesda Research Laboratories) and cloned by transformation into strain DH1 by the calcium chloride method as described elsewhere (5).

Recombinant plasmids were prepared from 100-ml cultures by the rapid boiling method (14) or from liter cultures by the ethidium bromide-cesium chloride equilibrium centrifugation method (4).

RESULTS

The principles and details of the tRNA suppressor rescue method have recently been published elsewhere (Shimizu et al., submitted for publication) and should be consulted for a full description of the derivation and construction of the *E. coli* tRNA SupF plasmid pK5 and the double-amber derivatives of the λ1059 and λL47 cloning vectors. This report will describe briefly

the initial strategy for the isolation of the CHO TK gene and then describe in detail only those experiments that led directly to the TK gene cloning.

High-molecular-weight DNA from the A-29 cell line of CHO cells (8) was digested with either *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, or *Hin*dIII and transfected as a calcium-phosphate precipitate onto mouse Ltk$^-$ cells to assay for hamster TK transformation activity as described previously (42). Only those A-29 DNAs digested with *Bam*HI or *Bcl*I retained TK transformation activity. This activity in *Bam*HI digests of A-29 was analyzed further by sucrose gradient fractionation and shown to be contained in a 19- to 25-kilobase (kb) fraction; the activity in *Bcl*I digests was contained in a 15- to 18-kb fraction. The transformation-active gradient fractions from either *Bam*HI or *Bcl*I digests were subsequently ligated in an approximately equimolar ratio to the 1.5-kb *Bam*HI fragment of pK5 containing an *E. coli* tRNA *supF* gene (Fig. 1) and transfected onto mouse Ltk$^-$ cells. A total of 11 primary TK$^+$ transformants were isolated from such transfections. Eight transformants were obtained from transfections with *Bcl*I/pK5-ligated DNA, and the remainder were obtained from transfections with *Bam*HI/pK5 DNA. Genomic DNA was isolated from mass cultures of each primary transformant, digested with *Eco*RI, and analyzed by Southern blotting with a nick-translated tRNA SupF DNA probe.

Since it was known from earlier work (43) that transfected cells which incorporate a selectable DNA sequence incorporate large amounts of extraneous DNA as well, it was not surprising to find that all 11 primary DNAs contained multiple copies of the *E. coli* tRNA SupF DNA marker. It was impossible to know, however, on the basis of this blotting data alone, whether any of the tRNA *supF* genes in these primary DNAs were ligated directly to the *Bam*HI or *Bcl*I fragment containing the hamster TK gene. To test for such a linkage and to purge the primary DNAs of unlinked SupF DNA, we used the DNA from each primary transformant as a hamster TK gene donor in a second round of transfections onto mouse Ltk$^-$ cells, to generate from each primary DNA a family of six secondary transformant colonies.

Secondary transformants from each primary DNA were expanded independently, and genomic DNA was prepared from each. These DNAs were digested with *Eco*RI (which cuts once within the 1.5-kb *Bam*HI SupF fragment of pK5) and Southern blotted with a SupF DNA probe (Fig. 1). Of the 11 families of secondary DNAs, 7 contained at least one member with an *Eco*RI fragment reactive with the SupF probe. Two of these seven DNA families, moreover, showed a similar pattern of *supF*-reactive *Eco*RI fragments, which was interpreted in the following straightforward manner. All secondary DNAs derived from the C52 and 2C1 primary DNAs. (*Bcl*I digests) contained two *Eco*RI fragments reactive with the SupF DNA probe (Fig. 1). One of these fragments, 3.5 kb in size, was common to all secondary DNAs derived from either the C52 or 2C1 primary DNAs. The other *Eco*RI fragment, however, varied in size and distinguished C52 from 2C1 secondary DNAs. To account for the common 3.5-kb *Eco*RI fragment, it was assumed that both C52 and 2C1 primary DNAs contained a tRNA *supF* gene ligated in precisely the same orientation to the same end of the *Bcl*I hamster TK gene fragment. The common 3.5-kb *Eco*RI fragment was assumed, therefore, to span the *Eco*RI site within the *supF* gene to the nearest *Eco*RI site within the *Bcl*I TK gene fragment, and the other pK5-

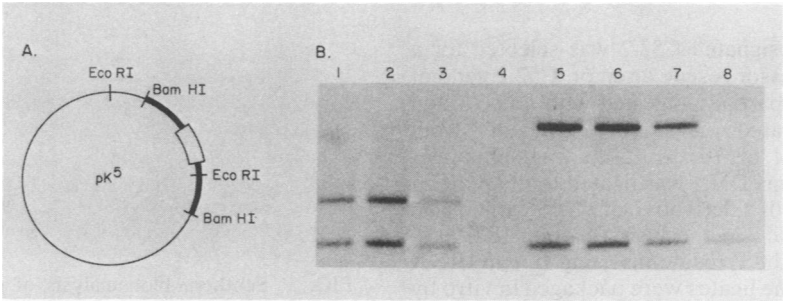

FIG. 1. (A) Map of tRNA *supF*-containing plasmid pK5. The open box indicates the approximate location of the tRNA *supF* gene coding sequences. The light lines indicate pBR322 sequences; the heavy lines indicate DNA sequences flanking the tRNA *supF* gene in pBR322. (See Shimizu et al. [submitted for publication] for a full description of pK5.) (B) Southern blot analysis of pK5-reactive *Eco*RI fragments in digests of secondary mouse transformant DNAs derived from the primary DNAs C52 and 2C1. The filters were probed with nick-translated pK5 DNA. Lane 1, 2C1.3; lane 2, 2C1.4; lane 3, 2C1.7; lane 4, mouse Ltk$^-$ DNA; lane 5, C52.2; lane 6, C52.7; lane 7, C52.9; lane 8, mouse Ltk$^-$ DNA reconstructed with 10 ng of DNA from λ52.

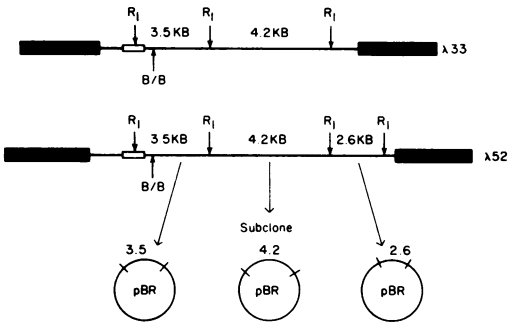

FIG. 2. *Eco*RI restriction maps of the recombinant phage isolates λ33 and λ52 derived from the C52.2 secondary DNA by the tRNA rescue suppressor method. The open boxes indicate *Eco*RI fragments reactive with a tRNA SupF DNA probe. The heavy lines represent λ1059 *Sam*7 sequences.

reactive *Eco*RI fragment was assumed to span the *Eco*RI site within the *supF* gene to an *Eco*RI site in genomic DNA flanking the *Bcl*I TK gene fragment. To strengthen this interpretation, we digested DNAs from the C52 and 2C1 secondary DNA families with *Hin*dIII and analyzed them by blotting with the SupF probe. As expected, all secondary C52- and 2C1-derived DNAs contained two *supF*-reactive fragments, one of which was common to all secondary DNAs analyzed (data not shown).

The linkage of this tRNA *supF* gene to the *Bcl*I TK gene fragment in the C52 and 2C1 primary DNAs, moreover, was sufficiently close to hamster TK structural gene sequences that the SupF DNA was cotransfected with TK gene through multiple secondary transfection events. It seemed likely, therefore, that in a biological rescue of the tRNA *supF* gene from any C52- or 2C1-derived secondary DNA, the hamster TK gene or fragments of it could be simultaneously recovered in DNA flanking the tRNA *supF* gene.

A DNA designated C52.2 was selected for a tRNA suppressor rescue attempt. C52.2 genomic DNA was partially digested with *Sau*-3A and size fractionated on sucrose gradients from which a pool of 10- to 15-kb fragments was recovered. This DNA was ligated to the purified *Bam*HI arms of a derivative of λ1059 carrying an amber mutation in the lysis function (S7) suppressible by the tyrosine-inserting *E. coli* tRNA *supF* gene. The ligates were packaged in vitro by the method of Hohn (12), titrated first on *supF*$^+$-containing *E. coli* BNN45, and then used to infect lawns of SupF$^-$ *E. coli* KS624 on which only amber-revertant phage and those recombinant phage incorporating an *E. coli supF* gene from the secondary DNA can plaque. Using this rescue method, we isolated four recombinant phage each containing a *supF* gene by screening approximately 4×10^6 recombinant λ1059 *Sam*7 phage. The DNAs obtained from liquid-lysate cultures of each of the four isolates lacked TK-transforming activity, however, when transfected onto mouse Ltk$^-$ cells.

These four isolates were subsequently digested with *Eco*RI, Southern blotted, and analyzed with a SupF DNA probe. Two of the four, designated λ33 and λ52, contained a 3.5-kb *Eco*RI fragment which hybridized with the tRNA SupF probe and comigrated with the 3.5-kb *Eco*RI fragment common to all secondary transformant DNAs generated with either the C52 or 2C1 primary DNA (Fig. 2). The 4.2- and 2.6-kb *Eco*RI fragments adjacent to the conserved 3.5-kb fragment in λ52 failed to react with the tRNA SupF DNA probe and, therefore, were presumed to derive from the *Bcl*I hamster TK gene fragment and to contain portions of the TK gene itself. To test this assumption, we subcloned the 4.2- and 2.6-kb *Eco*RI fragments, into pBR322 and used them as hybridization probes against a panel of secondary DNAs derived from independent primary DNAs, as well as CHO A-29 and mouse Ltk$^-$ DNAs (Fig. 3). These 4.2- and 2.6-kb *Eco*RI probes detected homologous *Eco*RI fragments in all secondary DNAs tested and in A-29 CHO DNA, although neither fragment was detected in mouse Ltk$^-$

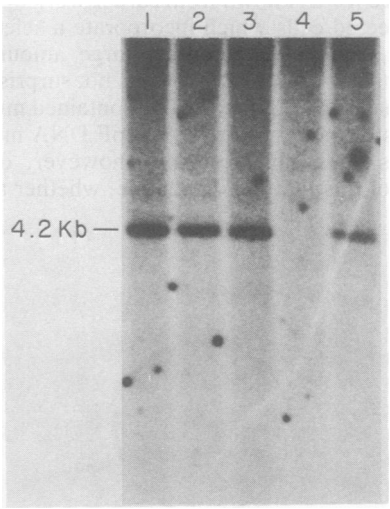

FIG. 3. Southern blot analysis of genomic DNA from secondary mouse transformant cell lines probed with the 4.2-kb *Eco*RI fragment of λ52. Genomic DNA (10 μg) was digested with *Eco*RI, electrophoresed through 1.0% agarose gels, and transferred to nitrocellulose filters (34). Each secondary DNA was derived from a different primary DNA. Lane 1, 2T1.2; lane 2, 2T3.2; lane 3, 2T2.7; lane 4, mouse Ltk$^-$; lane 5, CHO A-29 DNA.

DNA. At this point, the TK gene cloning strategy was modified to take advantage of the 4.2- and 2.6-kb *Eco*RI fragments obtained λ52 by the suppressor rescue method. It was reasoned that these *Eco*RI fragments could be used as hybridization probes to screen a recombinant phage library of A-29 DNA constructed by ligating *Bcl*I fragments to a *Bam*HI-accepting phage vector. Any recombinant phage identified by hybridization with the 4.2- and 2.6-kb probes would, therefore, be expected to contain the entire structural sequences of the hamster TK gene.

To accurately size the *Bcl*I TK fragment in A-29 DNA before sucrose gradient enrichment, a *Bcl*I digest of A-29 was electrophoresed through a 0.7% agarose gel containing high-molecular-weight DNA markers, Southern blotted, and probed with the 4.2- and 2.6-kb *Eco*RI subclones. This experiment revealed that the *Bcl*I TK gene fragment was, at a minimum, 18 kb in size and, therefore, likely to lie above the threshold of acceptance as a recombinant insert in any of the *Bam*HI-accepting phage vectors then available. Since it was known that rearrangements of genomic DNA flanking selectable DNA sequences can occur through multiple rounds of transfection, *Bcl*I digests of a number of secondary DNAs from the C52 primary DNA were similarly analyzed in the expectation that one or several might contain the 4.2- and 2.6-kb *Eco*RI sequences on a *Bcl*I fragment less than 18 kb in size. This expectation was fulfilled in the secondary DNA C52.9, which contained a 14-kb *Bcl*I fragment reactive with the 4.2- and 2.6-kb probes. This DNA was, therefore, digested with *Bcl*I, size fractionated into a 10- to 15-kb pool, and ligated purified *Bam*HI arms of the phage vector λL47. This ligate was packaged in vitro, and recombinant phage were plaqued onto lawns of *E. coli* BNN45 and screened by hybridization with the 4.2- and 2.6-kb *Eco*RI subclones as probes. A single recombinant phage, designated λ34S4, which reacted strongly with the hybridization probe, was recovered from a screening of 4×10^5 recombinant phage. The DNA from liquid cultures of λ34S4 was transfected onto mouse Ltk$^-$ cells and was highly active in transforming mouse Ltk$^-$ cells to Tk$^+$.

The Tk transformation efficiency of the λ34S4 phage and the restriction enzyme sensitivity of its transformation activity are shown in Table 1. Under these experimental conditions, λ34S4 transforms mouse Ltk$^-$ cells with an efficiency of 8 to 14 colonies per ng, roughly equivalent to the efficiency reported previously for the cloned chicken TK gene (29). As expected from the initial restriction enzyme sensitivity profile of the hamster TK gene defined with A-29 DNA, the λ34S4 transformation activity was retained after *Bam*HI digestion, but abolished by digestions with *Bgl*II, *Eco*RI, and *Hin*dIII. The restriction sites for these and other enzymes reported in Table 1 were mapped to λ34S4 in an attempt to define those sequences within the 13.2 kb of genomic DNA in λ34S4 essential for its Tk transformation activity (Fig. 4). At its right end, λ34S4 contains 3.0 kb of genomic DNA included in a 5.5-kb *Eco*RI junction fragment with λL47 right-arm sequences. This *Eco*RI fragment hybridizes strongly to the 3.5-kb subclone from λ52 and is colinear with this subclone through the *Xho*I and *Hpa*I sites previously mapped to the 3.5-kb *Eco*RI subclone. The 5.5-kb *Eco*RI fragment does not, however, hybridize with a tRNA SupF DNA probe. This result was, at first, unexpected since C52.9 DNA contains the 3.5-kb *Eco*RI fragment common to C52- and 2C1-derived secondary DNAs. It was later rationalized, however, by demonstrating that the SupF DNA sequences could be cut from the 3.5-kb *Eco*RI fragment of λ52 by digestion with *Bcl*I. Since pK5 does not contain a *Bcl*I site, it has been assumed that the original *Bcl*I-*Bam*HI junction between the hamster TK gene fragment and the 1.5-kb *Bam*HI pk5 fragment can, for some reason, be recut with *Bcl*I.

λ34S4 contains the 4.2- and 2.6-kb *Eco*RI fragments present in the λ52 phage isolated by suppressor rescue. Since λ52 was inactive in mouse Tk$^-$ transformation assays, whereas λ34S4 was active, sequences to the left of the 2.6-kb *Eco*RI fragment must contain hamster TK gene sequences. Hamster TK structural gene sequences must be contained within the

TABLE 1. λ34S4 Ltk$^-$/Ltk$^+$ transformation[a]

DNA	Amt (ng)	Restriction enzyme	Colonies
λ34S4	3	Uncut	43
λ34S4	30	Uncut	240
λ34S4	150	*Bam*HI	>600
λ34S4	150	*Bgl*II	0
λ34S4	150	*Eco*RI	0–3
λ34S4	150	*Hin*dIII	0
λ34S4	150	*Hpa*I	>600
λ34S4	150	*Kpn*I	0–5
λ34S4	150	*Pvu*II	0–4
λ34S4	150	*Sal*I	0–10
λ34S4	150	*Sma*I	0–4
λ34S4	150	*Xho*I	>600

[a] λ34S4 DNA was purified from virions banded to equilibrium in CsCl (46). The DNAs were digested at enzyme/mass ratios of 3 U/μg or greater for 4 h at 37°C. Phage DNA was transfected onto mouse Ltk$^-$ cells in the presence of 20 μg of genomic DNA per ml from mouse Ltk$^-$ cells. Colonies were counted after 17 days of HAT selection (42). The colony count data are derived from two to three replica experiments.

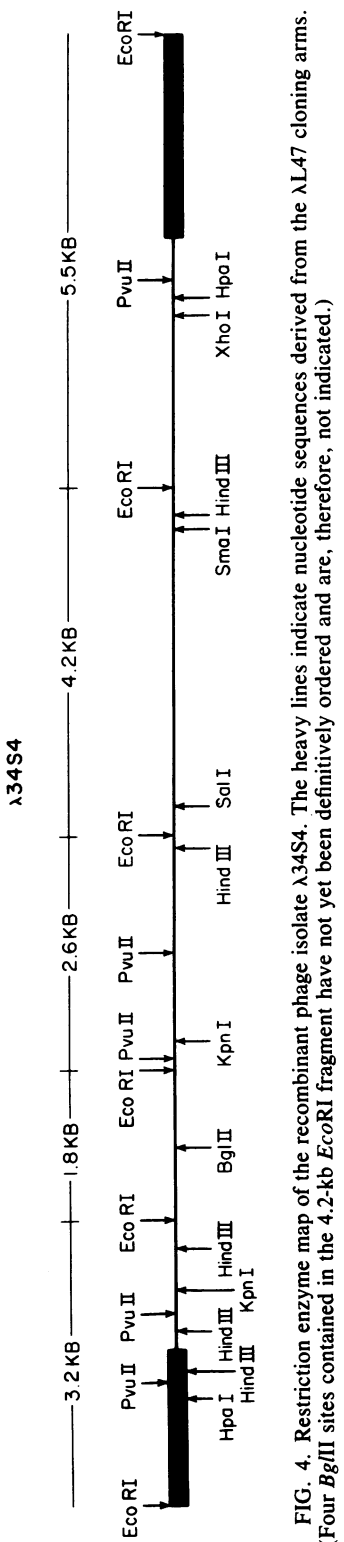

FIG. 4. Restriction enzyme map of the recombinant phage isolate λ34S4. The heavy lines indicate nucleotide sequences derived from the λL47 cloning arms. (Four *Bgl*II sites contained in the 4.2-kb *Eco*RI fragment have not yet been definitively ordered and are, therefore, not indicated.)

2.6- and 4.2-kb *Eco*RI fragments as well, since both *Sal*I and *Sma*I, which cut within the 4.2-kb *Eco*RI fragment, abolish the Tk transformation activity of λ34S4. Since the *Sma*I site in the 4.2-kb fragment lies 800 base pairs to the left of the 5.5-kb *Eco*RI fragment, the hamster TK gene must extend over a minimum of 6.2 kb in λ34S4. At the present time, the boundary of hamster TK gene sequences to the left of the 2.6-kb *Eco*RI fragment has not yet been defined through the restriction enzyme analysis of λ34S4 transformation activity.

Since the rearrangement of hamster DNA sequences in C52.9 DNA must have occurred at this end of the TK gene fragment, it was important to the issue of TK gene boundaries in λ34S4 to provide some measure of the colinearity of hamster DNA sequences at the left end of the clone with genomic A-29 DNA. *Pvu*II digests of λ34S4 and A-29 DNAs were, therefore, Southern blotted and probed with the 3.2-kb *Eco*RI fragment (data not shown). This experiment established that the λ34S4 to the left of the 2.6-kb *Eco*RI fragment is colinear with A-29 genomic DNA, at least from the rightmost *Pvu*II site in the 3.2-kb *Eco*RI fragment through the leftmost *Pvu*II site within the 2.6-kb *Eco*RI fragment (data not shown). Hamster TK gene sequences, therefore, may be contained within both the 1.8- and 3.2-kb *Eco*RI fragments.

The λ34S4 clone has no nucleotide sequence homology with the herpes simplex virus type 1 (HSV-1) TK gene detectable by moderate-stringency hybridization with a nick-translated HSV TK probe, although it does show limited homology with the cloned chicken TK gene (Fig. 5). The homologies detected are to the 3.2-kb *Eco*RI junction fragment at the left end of λ34S4 and to the 4.2-kb *Eco*RI fragment as well. This homology is apparent after a final wash at 68°C in 50 mM NaCl and, on the basis of this high stringency, is presumed to reflect a significant nucleotide sequence conservation between the two TK genes. If the sequences in the leftwardmost 3.2-kb *Eco*RI junction fragment of λ34S4, which hybridize with the chicken TK gene, are, in fact, part of the same TK gene transcription unit defined by *Sma*I digestion, the hamster TK gene must extend over a minimum of 8.5 kb. It is important to point out that since the 3.2-kb *Eco*RI fragment does not hybridize with the 4.2-kb *Eco*RI fragment (data not shown), these two *Eco*RI fragments cannot be considered as tandemly linked TK genes or TK gene fragments.

Identification of cytoplasmic hamster TK mRNA. With hamster TK structural gene sequences in hand, experiments were undertaken to identify the hamster TK mRNA in cytoplasmic RNA preparations and to provide direct experimental support for the assumption that the

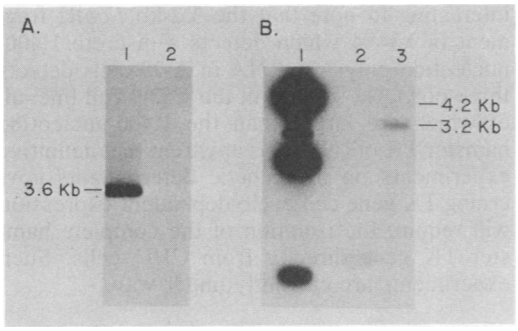

FIG. 5. Nucleotide sequence homology studies of the λ34S4 isolate with the TK gene of HSV-1 and the chicken TK gene. (A) Equimolar amounts of an *Eco*RI digest of λ34S4 and the 3.6-kb *Bam*HI fragment of HSV-1 containing the HSV TK gene were electrophoresed through 1.0% agarose gels and transferred to a nitrocellulose filter (Shimizu et al., submitted for publication). The filter was probed with nick-translated HSV-1 TK DNA and washed to a final stringency of 50 mM NaCl at 68°C. Lane 1, purified *Bam*HI fragment of HSV-1; lane 2, *Eco*RI digest of λ34S4. (B) Equimolar amounts of an *Eco*RI digest and *Hin*dIII digest of λ34S4 and an *Eco*RI-*Hin*dIII digest of pCHTK5, containing the chicken TK gene on a 2.3-kb *Eco*RI-*Hin*dIII fragment (29), were electrophoresed through a 1% agarose gel and transferred as described above. The filter was probed with nick-translated pCHTK5 DNA and washed to a final stringency of 50 mM NaCl at 68°C. Lane 1, *Eco*RI-*Hin*dIII digest of pCHTK5; lane 2, *Hin*dIII digest of λ34S4; lane 3, *Eco*RI digest of λ34S4.

growth dependence of TK gene expression is governed in a major way by the level of cytoplasmic TK mRNA. Since the λ34S4 clone, as a nick-translated probe, reacted with an unexpected number of RNA species in cytoplasmic poly(A)$^+$ preparations from asynchronously growing A-29 cells, RNA blotting experiments were repeated systematically using each *Eco*RI subclone of λ34S4 as a probe. The results of such an experiment with the 3.2-kb subclone are shown in Fig. 6. This probe reacts with a single poly(A)$^+$ species, approximately 1,400 nucleotides in size. This RNA species is not detected with the 1.8-kb *Eco*RI subclone, which reacts predominantly with a population of heterogeneously sized small RNAs which copurify with poly(A)$^+$ RNA, nor is it detected with the 2.6-kb *Eco*RI subclone. A 1,400-nucleotide species is, however, detected in Northern blots probed with the 4.2-kb *Eco*RI subclone which, like the 3.2-kb *Eco*RI fragment, shares homology with the chicken TK gene. This 4.2-kb subclone detects other higher-molecular-weight RNA species as well. These RNAs may simply be detected through moderately repeated DNA sequences contained within the 4.2-kb *Eco*RI fragment which are common to other mature hamster mRNAs.

Since both λ34S4 *Eco*RI fragments which show homology with the chicken TK gene react with a 1,400-base-pair mRNA in A-29 cells, this 1,400-nucleotide poly(A)$^+$ RNA has been tentatively identified as the hamster TK mRNA. This interpretation is supported indirectly by a Northern blot analysis of cytoplasmic poly(A)$^+$ RNA from mid-log cultures of CHO Tk$^-$ cells (10). Although the 3.2-kb *Eco*RI subclone of λ34S4 detects a 1,400-nucleotide RNA species in poly(A)$^+$ RNA from CHO Tk$^-$ cells, this RNA species is less than 1/20 as abundant as the 1,400-nucleotide species in A-29 RNA.

The concentration of the 1,400-nucleotide hamster TK mRNA is dramatically reduced in poly(A)$^+$ RNA prepared from stationary-phase confluent cultures of A-29 cells (Fig. 6). This

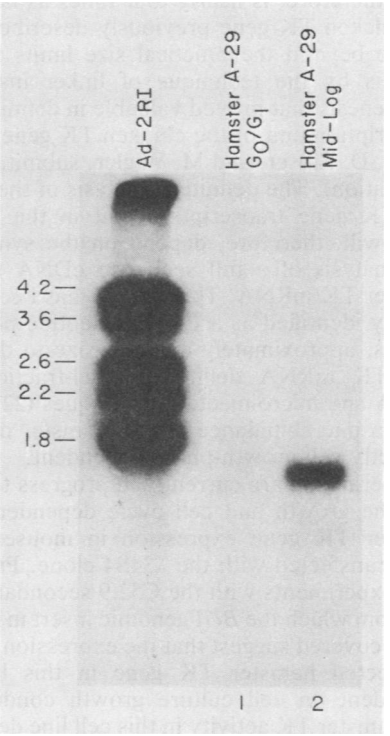

FIG. 6. RNA blotting analysis of cytoplasmic poly(A)$^+$ RNA isolated from CHO A-29 cells grown under various culture conditions. Poly(A)$^+$ RNA (3 μg) was denatured in formamide formaldehyde and electrophoresed through 1.8% agarose formaldehyde gel (20). The RNA was transferred to nitrocellulose filters (38) and probed with nick-translated 3.2-kb *Eco*RI DNA from λ34S4. Molecular weight markers are an *Eco*RI digest of adenovirus type 2 DNA. Lane 1, Poly(A)$^+$ RNA from stationary-phase cultures of A-29 cells; Lane 2, poly(A)$^+$ RNA from mid-log cultures of asynchronously growing A-29 cells.

result constitutes the first direct experimental evidence to support the notion that the growth dependence of hamster TK gene expression is significantly regulated at the level of cytoplasmic TK mRNA. Both poly(A)$^+$ RNA preparations from Fig. 6 were analyzed in parallel with a mouse actin DNA probe and shown to be physically undegraded.

DISCUSSION

This cloning of the full structural gene sequences of the CHO TK gene constitutes the first step in a projected study of the genetic determinants governing the cell cycle-dependent expression of this gene. Based on restriction enzyme sensitivity data, RNA blotting data, and nucleic acid homology studies with the cloned chicken TK gene, these structural gene sequences extend over 8.5 of the 13.2 kb of genomic DNA in the λ34S4 clone. The CHO TK gene, therefore, is nearly four times as large as the chicken TK gene previously described (29) and so beyond the practical size limits for an analysis by the technique of linker insertion mutagenesis that proved valuable in defining the transcription unit of the chicken TK gene (T. J. Kwoh, D. Zipser, and M. Wigler, submitted for publication). The definitive analysis of the hamster TK gene transcription unit in the λ34S4 clone will, therefore, depend on the synthesis and analysis of a full sequence cDNA to the hamster TK mRNA. This mRNA has been tentatively identified as a 1,400-nucleotide poly(A) species, approximately equal in size to the human TK mRNA defined by gel-fractionated mRNA and microinjection techniques (22). The cytoplasmic abundance of this hamster mRNA is strictly cell-growth-phase dependent.

Experiments are currently in progress to analyze the growth and cell cycle dependence of hamster TK gene expression in mouse Ltk$^-$ cells transfected with the λ34S4 clone. Preliminary experiments with the C52.9 secondary cell line from which the *Bcl*I genomic insert in λ34S4 was recovered suggest that the expression of the transfected hamster TK gene in this line is dependent on cell culture growth conditions. The hamster TK activity in this cell line declines as the cells reach stationary confluence and rest in G0 without serum refreshment, although the rate of this decline is less dramatic than the decline of mouse TK activity when Ltk$^+$ cells are grown under similar metabolic conditions. It is conceivable that the rearrangements of genomic DNA flanking the hamster TK structural gene sequences in λ34S4 have altered or replaced normal controlling elements which govern the transcription or processing of hamster structural gene sequences. In this regard, it is interesting to note that the 3.2-kb *Eco*RI fragment of λ34S4 which detects a discrete 1,400-nucleotide poly(A)$^+$ RNA in A-29 cells detects three poly(A)$^+$ species in the C52.9 cell line, all of which are larger than the 1,400-nucleotide hamster TK mRNA. It is apparent that definitive experiments on the genetic determinants governing TK gene cell cycle-dependent expression will require the isolation of the complete hamster TK gene directly from CHO cells. Such experiments are currently under way.

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