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Expression of the herpes thymidine kinase gene in Xenopus laevis oocytes: an assay for the study of deletion mutants constructed in vitro

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ABSTRACT

When Xenopus laevis oocyte nuclei are injected with a recombinant plasmid containing the Herpes Simplex Virus (HSV) thymidine kinase (tk) gene, a 100-fold increase in tk enzymatic activity is observed. Three lines of evidence show that this increase in tk activity is a result of the expression of the HSV tk gene. First, the enzymatic activity is selectively inactivated by the IgG fraction of antiserum raised against HSV tk protein. Second, a polypeptide that comigrates with authentic HSV tk on polyacrylamide gels is synthesized uniquely by oocytes injected with the HSV tk gene. Third, the induced tk activity found in injected oocytes is capable of phosphorylating deoxycytidine, a substrate that is utilized by HSV tk but not by cellular tk. We have used these observations to establish an assay for examining the activity of mutated variants of the HSV tk gene. Two sets of deletion mutants of the tk gene were constructed in vitro. In one set varying amounts of 5' flanking and intragenic sequences are deleted. The other set is deleted at the 3' end of the gene. By testing the activity of each mutant in the oocyte injection assay we have delimited functional boundaries corresponding to the 5' and 3' termini of the HSV tk gene.

INTRODUCTION

The Herpes Simplex Virus (HSV) genome includes a gene for thymidine kinase (tk) that is different from cellular tk (Dubbs and Kit, 1964). The viral tk gene is located within a 3.4 kilobase (kb) DNA fragment that can be isolated from the HSV genome by Bam HI restriction enzyme digestion. In an elegant series of reports Axel and colleagues have shown that this isolated 3.4 kb tk DNA fragment can be used to stably transfect tk^- cells to the tk^+ genotype (Wigler et al., 1977; Pellicer et al., 1978).

We and others have introduced the 3.4 kb Bam HI fragment into the prokaryotic plasmid pBR-322 (McKnight et al., 1979; Colbere-Garapin et al., 1979; Wilkie et al., 1979; Enquist et al., 1980). The cloned tk gene retains the capacity to transfect tk^- cells. With an isolated gene and an assay for its expression, it should be possible to delimit the functional components of the gene by deleting it systematically in vitro and then testing the effects of

of specific alterations on gene expression. While this approach can be adapted to the HSV tk gene using the transfection assay, the assay requires 2-3 weeks before positive cell colonies can be scored. For this reason we microinject the HSV tk gene into amphibian oocytes and assay for tk enzymatic activity.

It is well established that Xenopus oocytes are capable of synthesizing large quantities of 5S and tRNA in response to microinjection of 5S DNA and transfer DNA, respectively (Brown and Gurdon, 1977; Gurdon and Brown, 1978; Kressman et al., 1978a; DeRobertis and Olson, 1979; Melton and Cortese, 1979). Both 5S and tDNA are expressed by RNA polymerase III, which is in abundant supply in mature oocytes (Roeder, 1974). Genes expressed by the form I and II RNA polymerases, however, elicit more modest responses when injected into oocytes. Due to the high level of endogenous rRNA synthesis, Trendelenburg and Gurdon (1978) resorted to an electron microscopic assay to document the accurate expression of ribosomal DNA, an event catalyzed by form I RNA polymerase. Regarding structural gene expression in microinjected oocytes, DeRobertis and Mertz (1977) and Rungger and Türler (1978) have shown that viral-specific capsid proteins and tumor antigen are expressed in oocytes injected with SV-40 DNA, indicating that functional SV-40 mRNA can be synthesized by oocytes. More recently, Probst et al. (1979) reported that oocytes injected with sea urchin histone DNA synthesize transcripts indistinguishable from the histone H2A and H2B mRNAs made in sea urchin embryos.

In the present study we demonstrate that Xenopus laevis oocytes synthesize active tk enzyme when microinjected with the HSV tk gene. We have used this assay to score the functional capacity of mutant tk genes constructed in vitro. The results of these experiments roughly delimit the 5' and 3' boundaries of the tk gene. In the accompanying report we identify these boundaries precisely by DNA sequencing and mRNA mapping.

MATERIALS AND METHODS

Oocyte Injections:

Mature oocytes were removed from Xenopus laevis females, dissected from somatic tissue and prepared for injection according to procedures modified from Gurdon (1974) and Kressman et al. (1978b). Individual stage 5-6 oocytes (Dumont, 1972) were aligned on nylon mesh affixed to the bottom of a 35 mm plastic petri dish, covered with Barth's buffer (Barth and Barth, 1959), as modified by Gurdon (1974), and centrifuged at 2,000 x G for 20 minutes. This procedure embeds the oocytes in the nylon mesh and displaces the germinal

vesicle (nucleus) to the top of the oocyte where it is clearly visible. For each experiment, batches of 40-60 oocytes were injected. Each oocyte was injected with 20-40 nl of a 0.2 mg/ml solution of supercoiled plasmid DNA in 10 mM Tris·HCl, pH 7.4/10 mM NaCl. In experiments designed to radio-label oocyte proteins, approximately 50 nCi of ^{35}S-methionine (977 Ci/mmole, New England Nuclear) was injected into each oocyte along with the appropriate DNA sample. In experiments designed to test the effects of α-amanitin (Calbiochem), the inhibitor was mixed with the DNA sample prior to injection.

Preparation and Enzyme Analysis of Oocyte Extracts:

Thymidine kinase enzyme assays were performed on extracts prepared from oocytes 24 hours following microinjection. The surviving oocytes of each batch (40-80% viability) were pooled, washed with phosphate buffered saline and homogenized in 10 µl/oocyte of a buffer consisting of 0.01 M Tris·HCl, pH 7.5/0.01 M KCl/1 mM $MgCl_2$/1 mM 2-mercaptoethanol /50 µM thymidine. Homogenates were centrifuged at 1000,000 x G for one hour at 4°C. The supernatant fraction was retrived and duplicate 20 µl samples were assayed for thymidine kinase enzyme activity according to Wigler et al. (1977). Immunoglobulin-mediated enzyme neutralization was carried out by mixing 25 µl of oocyte homogenate with 20 µg of either non-immune IgG (a gift of P. Gearhart) or anti-tk IgG (a gift of S. Silverstein and A. El Kareh). The mixtures were incubated at 37°C for 30 minutes, overnight at 4°C and then were centrifuged for 2 minutes in a Beckman microfuge B. Supernatant fractions were assayed for tk activity. ^{125}Iododeoxycytidine assays were kindly carried out by W. Summers according to published procedures (Summers and Summers, 1977).

SDS Gel Electrophoresis of Labeled Oocyte Proteins:

Oocytes injected with plasmid DNA and ^{35}S-methionine were incubated for 24 hours, homogenized in 10 µl/oocyte 150 mM NaCl/20 mM $NaPO_4$, pH 7.2/0.5% NP-40. The supernatant fraction of a 100,000 x G centrifugation was recovered and prepared for gel electrophoresis according to Laemmli (1970). Immune precipitation reactions were carried out by incubating 100 µl of the 100,000 x G supernatant with 20 µg IgG fraction of either non-immune or anti-tk antiserum. Twenty microliters of a slurry of protein-A sepharose (2 mg protein A/ml, Pharmacia) was added to the IgG/homogenate mixture. The samples were vortexed at 3 minute intervals for 12 minutes then washed four times with 0.6 M NaCl/0.02 M $NaPO_4$, pH 7.2 and twice with 0.02 M Tris·HCl, pH 7.2. The remaining protein-A sepharose was then boiled for 2 minutes in SDS sample buffer prior to electrophoresis. Homogenate fractions and immune precipitates

were electrophoresed on 10% SDS polyacrylamide gels according to Laemmli (1970). Gels were stained with coomassie brilliant blue, photographed to locate molecular weight marker proteins, fluorographed with "Enhanse" (New England Nuclear), dried and exposed to preflashed Kodak XR film at -80^{o} for 3-7 days.

Construction of tk Deletion Mutants:

The thymidine kinase gene used in this and the accompanying study was isolated from HSV I macroplaque strain by recombining a 3.4 kb Bam HI fragment of viral DNA with Bam HI linearized pBR-322 (McKnight et al., 1979). The recombinant molecule, pHSV-106, contains the 3.4 kb Bam HI fragment of the viral DNA inserted at the Bam HI site of pBR-322 in the orientation shown in Figure 1. To produce deletion mutants progressing from the leftward portion of the inserted HSV DNA, pHSV-106 was digested to completion with Hind III (a gift of Y. Suzuki). Roughly 2 pmole of the linear molecules were digested with exonuclease III (Bethesda Research Laboratories) at 30^{o}C in a 50 µl reaction according to conditions described by Sakonju et al. (1980). 2.5 µl aliquots were removed to 4^{o}C at 30 second intervals. These fractions were pooled and digested with S_1 nuclease (Boehringer) in a 250 µl reaction carried out in 50 mM sodium acetate-acetic acid, pH 4.5/150 mM NaCl/0.5 mM $ZnSO_4$ using 10 units of S_1 nuclease (Vogt, 1973). The reaction was incubated at 25^{o}C for 30 minutes, placed in an ice bath for 30 minutes then stopped by the addition of 60 µl 0.5 m Tris·HCl, pH 9.5/100 mM EDTA. The sample was phenol extracted twice, ether extracted 4 times and ethanol precipitated. Hind III linker molecules (Heynecker et al., 1976) labeled with γ-^{32}P-ATP and polynucleotide kinase (Weiss et al., 1976) were ligated to deleted tk fragments in a 30 µl reaction containing 60 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/5 mM dithiothreitol/10 mM ATP and 2 units T_4 ligase (Bethesda Research Labs). A 20-fold molar excess of linker molecules was used and the reaction was incubated at 4^{o}C for 18 hours at which time the reaction was stopped by ethanol precipitation. The molecules were resuspended in 50 µl of 50 mM NaCl/6 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$, and digested with 10 units Hind III and 2 units Bam HI for 2 hours at 37^{o}C. The resulting DNA was loaded directly onto a 1% agarose gel cast in 40 mM Tris-acetic acid, pH 7.8/20 mM sodium acetate/2 mM EDTA. The gel was electrophoresed at 50 volts for 14 hours then autoradiographed. DNA molecules that migrated in the 1.5-3.4 kilobase range, as determined from the positions of molecular weight markers, were recovered from the gel according to the procedure of Tabak and Flavell (1978). This DNA was restricted a second time with 2 units Hind III then inserted into Bam HI/Hind III restricted pBR-322 vector DNA. Ligated molecules were used to transform E. coli HB-101 as described by Cohen et al.

(1972). Ampicillin-resistant, tetracycline-sensitive colonies were selected and the plasmid DNA of each was harvested by the procedures of Clewell and Helinski (1970) as modified by Sakonju et al. (1980). The approximate end point of each deletion mutant was estimated by restriction enzyme mapping. The exact endpoints of the deletion mutants were determined by DNA sequencing as described in the accompanying report (McKnight, 1980).

RESULTS

The HSV tk Gene is Expressed in Microinjected Oocytes:

Xenopus laevis oocytes were microinjected according to published methods (Gurdon, 1974; Kressmann et al., 1978b). Individual oocytes were injected with 20 - 40 nl of a 0.2 mg/ml solution of supercoiled plasmid DNA. The injected oocytes were incubated for 24 hours, homogenized, and centrifuged at 100,000 x G (Materials and Methods). The supernatant fraction was either assayed for tk enzymatic activity or electrophoresed on an SDS polyacrylamide gel.

The thymidine kinase gene used in these experiments consists of a 3.4 kb Bam HI restriction fragment isolated from HSV I that was ligated to the Bam HI site of pBR-322 (McKnight et al., 1979). Figure 1 is a diagram showing a limited restriction map of the recombinant plasmid, termed pHSV-106, and the polarity of tk gene transcription.

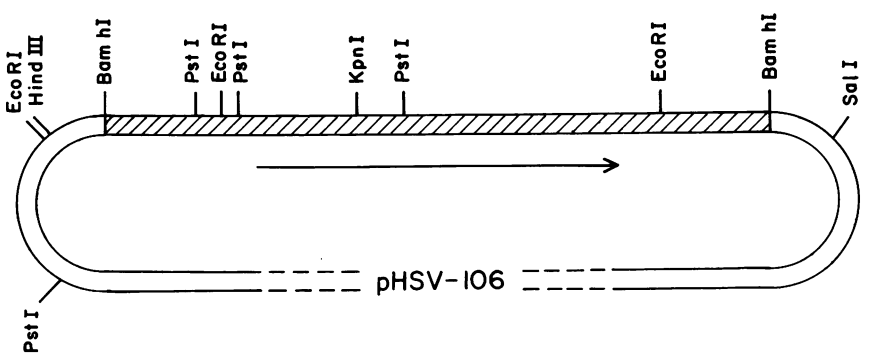

Fig. 1: Restriction Endonuclease Map of the Recombinant Plasmid pHSV-106.
The diagram shows a restriction endonuclease map of pHSV-106. The recombinant plasmid was constructed by inserting a 3.4 kb Bam HI fragment isolated from viral DNA (hashed area) into the Bam HI site within the tetracycline resistance gene of pBR-322. The arrow below the inserted DNA refers to the polarity of tk transcription.

The results of an experiment in which the tk enzymatic activity of oocytes injected with pHSV-106 was compared with that of uninjected oocytes and oocytes injected with pBR-322 are shown in Table I. As this experiment demonstrates, oocytes injected with the recombinant plasmid carrying the HSV tk gene contain approximately 175-fold more tk enzyme after a 24 hour incubation interval than do uninjected or mock-injected oocytes.

Identification of HSV Encoded Thymidine Kinase:

In order to determine whether the excess tk activity observed in oocytes injected with pHSV-106 is antigenically similar to authentic HSV tk protein, we pre-treated oocyte homogenates with the IgG fraction of antiserum raised against HSV tk. This IgG fraction is known to inactivate specifically HSV tk synthesized in tk^+ transfected cell lines (Pellicer et al., 1980). High speed supernatant fractions of oocytes injected with pHSV-106, uninjected oocytes and Xenopus laevis hepatocytes, were incubated with either anti-tk IgG or the IgG fraction purified from non-immunized rabbit serum (Materials and Methods). As Table II shows, pre-treatment of homogenates with anti-tk IgG neutralized the tk activity observed in oocytes that had been injected with pHSV-106. In contrast, the tk activity of extracts prepared from uninjected oocytes or Xenopus laevis liver tissue was not reduced by anti-tk IgG any more than by non-immune IgG. Moreover, the IgG fraction of non-immune serum does not affect the tk activity of extracts prepared from oocytes injected with pHSV-106. These results show that a substantial fraction of the tk activity observed in homogenates prepared from oocytes injected with tk DNA is specifically neutralized by anti-tk immunoglobulin. This indicates that the tk synthesized in oocytes is antigenically similar to authentic HSV tk.

TABLE I: THYMIDINE KINASE ENZYME ACTIVITY OF OOCYTE EXTRACTS

Source of Extract	tk Activity units x 10^{-2} per ml
uninjected oocytes	0.05
pBR-322-injected oocytes	0.06
pHSV-106-injected oocytes	8.82

Thymidine kinase assays were carried out by incubating 25 µl of a high-speed supernatant fraction of homogenized oocytes with ^{3}H-thymidine (see Materials and Methods). After 1 hour at 37°C the reaction mixes were spotted onto DEAE-81 filter discs, washed and counted by scintiallation spectrometry. The assay detects the conversion of thymidine, which does not adhere to DEAE, to thymidine monophosphate, which does adhere to the filter discs. One unit of thymidine kinase is defined as the amount of enzyme required to convert one nmole of thymidine to thymidine monophosphate per minute.

TABLE II. IMMUNOLOGICAL NEUTRALIZATION OF TK ENZYME ACTIVITY

Source of Extract	Uninjected oocytes	X. laevis hepatocytes	pHSV-106 injected oocytes
a) tk activity units x 10^{-2} per ml with PBS	0.041	0.720	3.330
b) tk activity units x 10^{-2} per ml with non-immune IgG	0.021	0.450	3.680
c) tk activity units x 10^{-2} per ml with anti-tk IgG	0.038	0.600	0.550
d) % residual tk activity ($^{c}/_{a}$ x 100)	97.3	83.3	16.5

Prior to tk enzyme assays, homogenate samples were incubated with one of three solutions: phosphate buffered saline, IgG fraction of non-immune serum or IgG fraction of anti-tk serum (see Materials and Methods). Incubations were carried out at 37°C for one hour, then at 4°C overnight. Samples were centrifuged for 5 minutes at 30,000 x G and the supernatant fractions were assayed for tk enzymatic activity.

We have analyzed by gel electrophoresis the proteins synthesized by oocytes coinjected with pHSV-106 and radio-labeled methionine. By separating and comparing the proteins synthesized by oocytes injected with pHSV-106 or with pBR-322 we hoped to identify the HSV tk polypeptide. Oocytes were co-injected with either pHSV-106 and ^{35}S-methionine, or pBR-322 and ^{35}S-methionine. Twenty-four hours later, the surviving oocytes were homogenized and prepared for SDS polyacrylamide gel electrophoresis (Materials and Methods).

HSV tk protein has an apparent molecular weight on SDS gels of 42,000 daltons (Summers et al., 1975; Cremer et al., 1978). The autoradiogram displayed in Figure 2 shows the distribution of labeled proteins synthesized in oocytes injected with either pBR-322 or pHSV-106. We were unable to discern any difference in the distribution of radiolabeled proteins of the two preparations in the 35-45,000 dalton range of the gel. We reasoned, however, that the tk protein band might be obscured by the relatively strong band of radioactive protein that migrates at an apparent molecular weight of 40,000 daltons. To resolve this problem, extracts prepared from injected oocytes labeled with ^{35}S-methionine were treated sequentially with either anti-tk IgG and protein-A sepharose, or non-immune IgG and protein-A sepharose (Materials and Methods). As the autoradiogram displayed in Figure 2 shows, homogenates

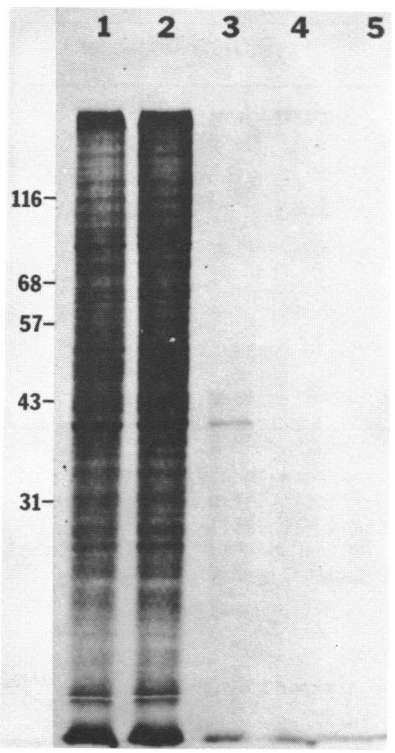

Fig. 2: Electrophoretic Resolution of Thymidine Kinase Protein Synthesized in Oocytes.

Batches of 100 oocytes were injected with ^{35}S-methione and either pHSV-106 or pBR-322 supercoiled plasmid DNA. Following a 24-hour incubation interval, the oocytes were homogenized and centrifuged at 100,000 x G. Proteins were separated on a 10% SDS polyacrylamide gel and detected by autoradiography. Lanes 1 and 2 show the distribution of polypeptides synthesized by oocytes injected with pBR-322 and pHSV-106 respectively. Lane 3 was loaded with proteins immunoprecipitated by anti-tk IgG from oocytes injected with pHSV-106. Lane 4 was loaded with proteins immunoprecipitated by anti-tk IgG from oocytes injected with pBR-322, and lane 5 was loaded with proteins immunoprecipitated by non-immune IgG from oocytes injected with pHSV-106. The single protein band observed in lane 3 co-migrates with thymidine kinase synthesized in cultured monkey cells infected with HSV I (data not shown). Numbers to the left of lane 1 mark the positions of molecular weight standard proteins that were visualized by coomassie staining.

prepared from oocytes injected with pHSV-106 contain a single radio-labeled protein that is immune-precipitated by anti-tk IgG. This protein, which has an apparent molecular weight of 40,000 daltons, is not precipitated by the IgG fraction of non-immune serum. Moreover, oocytes injected with pBR-322 do not produce a labeled protein that is precipitated by anti-tk IgG. The 40,000 dalton protein produced by oocytes injected with pHSV-106 co-migrates with a protein that is immune-precipitated by anti-tk IgG from extracts prepared from HSV infected cultured cells (data not shown). Thus, Xenopus laevis oocytes microinjected with pHSV-106 appear to synthesize authentic HSV thymidine kinase.

A third method of identifying the HSV tk enzyme takes advantage of the substrate specificity of HSV tk. 5-halo-deoxycytidines are substrates for phosphorylation by HSV tk but not by cellular tk (Cooper, 1973). Summers and

Summers (1977) have shown that HSV tk enzymatic activity can be assayed with ^{125}Iododeoxycytidine (IdC) either *in vivo* or *in vitro*. Since cellular tk is incapable of phosphorylating deoxycytidine, the IdC assay detects HSV tk in the presence of cellular tk activity. Homogenates prepared from oocytes injected with pHSV-106 contain enzymatic activity capable of phosphorylating iododeoxycytidine (Table III). While extracts prepared from HSV infected cells exhibit a similar activity, neither those from uninfected mouse cells nor from uninjected oocytes can phosphorylate iododeoxycytidine. These data, in concert with the results of immunological experiments and gel electrophoretic studies considered in the preceding sections, strongly support the conclusion that microinjected oocytes express the HSV tk gene.

Expression of HSV tk in Oocytes is Highly Sensitive to α-amanitin:

It is known that structural genes are transcribed by RNA polymerase form II. This enzyme is effecitvely inhibited by α-amanitin at a concentration of 0.1 μg/ml. In order to measure the concentration of α-amanitin required to block tk gene expression in frog oocytes, we injected tk DNA into oocyte nuclei along with varying concentrations of the transcription inhibitor and then assayed the oocytes for tk enzymatic activity. Since it is difficult to determine the final intranuclear concentration of α-amanitin in an injected oocyte, we first calibrated the α-amanitin dose response curve for 5S RNA synthesis. Varying amounts of α-amanitin were injected with α-^{32}P-GTP and recombinant 5S DNA, a gene known to be transcribed by RNA polymerase form III. Twelve hours after injecting oocytes with 5S DNA, RNA was isolated and electro-

TABLE III. ^{125}IODODEOXYCYTIDINE ASSAYS ON MOUSE CELL AND FROG OOCYTE EXTRACTS

Source of Extract	Protein Concentration	Cpm ^{125}I retained
Mouse L-cells	1.71 mg/ml	<250
HSV infected Mouse L-cells	1.43 mg/ml	16,437
Uninjected oocytes	2.30 mg/ml	535
pHSV-106 injected oocytes	2.11 mg/ml	11,725

Homogenates of HSV-infected and mock-infected mouse cell cultures were perpared according to Wigler *et al.* (1977). Oocyte homogenates were prepared for ^{125}IdC assays as described in Materials and Methods. ^{125}IdC assays, which measure the conversion of iododeoxycytidine to iododeoxycytidine-monophosphate were carried out according to published procedures (Summers and Summers, 1977). Protein concentrations were determined using Folins reagent according to Lowery *et al.* (1951).

phoresed on a thin polyacrylamide gel. As Figure 3A shows, 5S RNA is synthesized in oocytes injected with 5S DNA at α-amanitin concentrations up to 10 μg/ml. At higher α-amanitin concentrations little 5S RNA is synthesized. These results, which agree with a previous study (Gurdon and Brown, 1978), indicate that 5S RNA synthesis is inhibited by intermediate levels of α-amanitin.

Similar experiments were conducted to test the α-amanitin sensitivity of HSV tk gene expression in oocytes. Recombinant tk DNA was coinjected into oocyte nuclei with varying concentrations of α-amanitin. Twenty-four hours

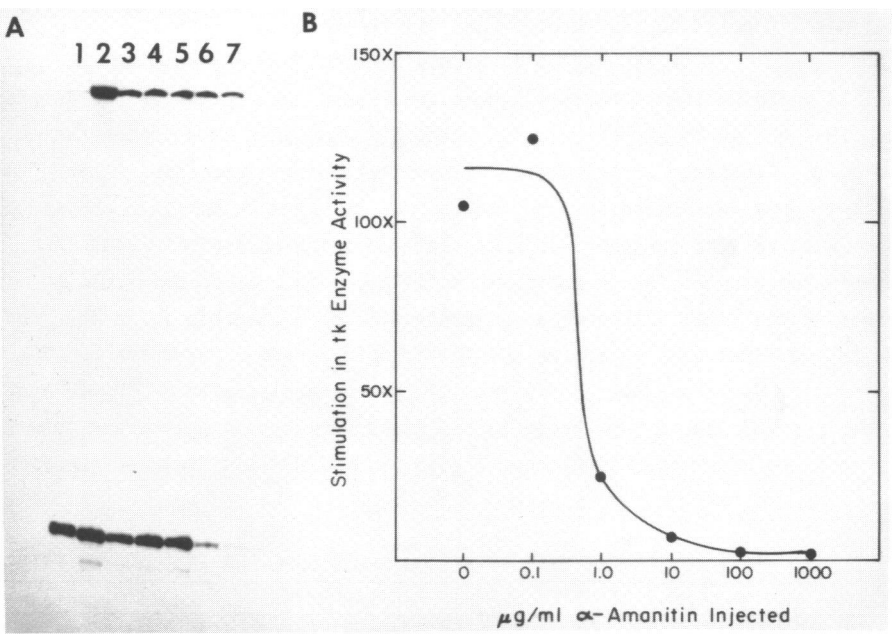

Fig. 3: Inhibitory Effects of α-amanitin on 5S and tk Gene Expression.
A) An autoradiograph of an 8% polyacrylamide sequencing gel that was used to size radio-labeled RNAs synthesized in oocytes injected with 5S DNA and varying concentrations of α-amanitin. 5S transcription reactions were carried out using a recombinant plasmid that contains a single repeat of Xenopus borealis somatic DNA (pXbs-1). Lane 1 was loaded with 5S RNA synthesized in vitro according to the procedures of Birkenmeier et al. (1978). Lanes 2-7 were loaded with RNA prepared from 10 oocytes injected with α-^{32}P-GTP, 10 ng pXbs-1 and 0, 0.1, 1.0, 10, 100 and 1000 μg/ml α-amanitin respectively. B) The α-amanitin inhibition curve for tk gene expression in microinjected oocytes. The oocytes used for the experiment shown in B were excised from the same female frog that was used for the experiment shown in (A). The data points at 0,0.1 and 1.0 μg/ml α-amanitin represent the average of duplicate experiments.

later homogenates of the injected oocytes were assayed for tk enzymatic activity. As Figure 3B shows, the synthesis of tk enzymatic activity is inhibited at 1.0 μg/ml α-amanitin. These results indicate that the expression of the HSV tk gene, as assayed by the appearance of enzymatically-active tk, is inhibited by roughly 1/100 the α-amanitin concentration needed to inhibit RNA polymerase form III, a result expected for a gene transcribed by RNA polymerase form II.

Construction and Characterization of Deletion Mutants of the tk Gene:

The coupled transcription/translation system of the amphibian oocyte provides a rapid and sensitive assay for the analysis of mutated variants of the HSV tk gene. Frog oocytes synthesize authentic HSV thymidine kinase enzyme via a transcriptional reaction that utilizes the RNA polymerase form expected of a structural gene. Moreover, the 5' terminus of the tk mRNA made in oocytes is identical to tk mRNA synthesized in HSV infected cells (McKnight, manuscript in preparation). We have used the oocyte assay to test the function of deletion mutants of the tk gene that were constructed *in vitro*. Two types of deletion mutants were prepared; one in which 5' sequences are deleted from the tk gene and another in which 3' sequences are deleted. A correlation between the structure of each deletion mutant and its capacity to function in the frog oocyte assay was expected to delimit the location of the tk gene on the 3.4 kb Bam HI fragment cloned in pHSV-106.

Transcription of the tk gene is known to occur from left to right as is diagrammed in Figure 1 (Smiley *et al.*, 1980; McKnight, 1980). A systematic set of 5' deletion mutants was prepared from pHSV-106 DNA that had been linearized with Hind III. Figure 4 diagrams the methods that were used to construct 5' deletion mutants of the HSV tk gene. The single Hind III site of pHSV-106 is 346 base pairs (bp) from the leftward Bam HI site (Sutcliffe, 1978). Molecules linearized with Hind III were digested with exonuclease III for varying lengths of time and made flush ended by digestion with S_1 nuclease. These shortened molecules were ligated to ^{32}P-labeled synthetic Hind III linkers, restricted with Bam HI and Hind III and electrophoresed on a 1% agarose gel. Deleted tk DNA fragments ranging in size from 3.4 to 1.5 kb were recovered and inserted into pBR-322 vector DNA that had been restricted with Bam HI and Hind III. Conditions for these steps were modified from those of Sakonju *et al.* (1980) as described in Materials and Methods. Recombinants were introduced into *E. coli* HB-101 and ampicillin-resistant, tetracycline-sensitive colonies were recovered. Eight 5' deletion mutants were chosen for study.

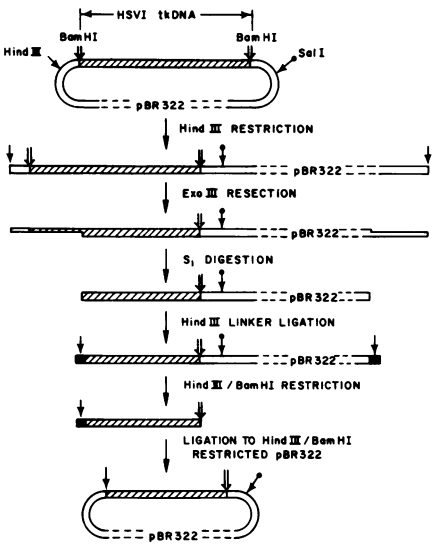

Fig. 4: Schematic Diagram of Methods used to Construct Deletion Mutants of the HSV tk Gene. Intact plasmid DNA was linearized with Hind III and subjected to digestion by exonuclease III for intervals varying from 1 to 10 minutes. The shortened molecules were made flush by S_1 nuclease digestion and synthetic Hind III linker molecules were ligated to the termini of each tk gene fragment. Ligated molecules were doubly restricted with Hind III and Bam HI and sized on a 1% agarose gel. DNA fragments ranging in length from 1.5 to 3.4 kb were recovered from the gel, inserted into Bam HI/Hind III cut pBR-322 vector DNA, and introduced into E. coli HB-101 by transfection. These procedures produced a set of 5' deletion mutants. 3' deletion mutants were constructed by the same procedures except that in the initial step the intact plasmid DNA was linearized with Sal I.

The endpoint for each of the mutants was estimated initially by restriction enzyme digestion and then determined exactly by DNA sequencing (McKnight, 1980).

A set of 3' deletion mutants of the HSV tk gene were constructed using the same procedure as described for the production of 5' deletion mutants. To produce 3' deletion mutants, however, the parental plasmid was linearized with Sal I, which cleaves the plasmid component of the recombinant at a position 275 bp from the rightward Bam HI site (Sutcliffe, 1978). 3' deletion mutants were characterized by restriction digestion and seven mutant isolates were chosen for study. The precise endpoints of four pertinent 3' deletion mutants were identified by DNA sequencing (McKnight, 1980).

Figure 5A shows photographs of two agarose electropherograms that were used to obtain estimates of the sizes of the fifteen deletion mutants selected for analysis. Figure 5b schematically diagrams the locations of the end points of these deletion mutants. The nomenclature for each mutant is

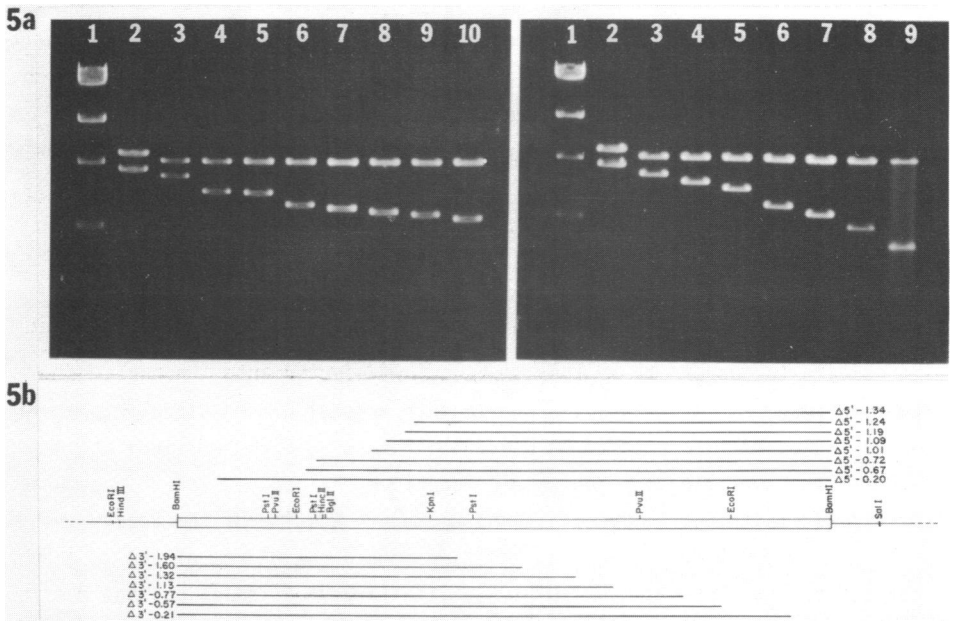

Fig. 5: Restriction Enzyme Analysis of 5' and 3' Deletion Mutants of the HSV Thymidine Kinase Gene.

A) Photographs of two agarose electrophoresis gels that were used to size restriction digests of tk deletion mutants. DNA fragments are visualized by ethidium bromide staining. Lane 1 of each gel was loaded with molecular weight standards (λ charon 4a restricted with Hind III) and lane 2 of each gel was loaded with pHSV-106 DNA that was restricted with Bam HI. The two bands resulting from Bam HI restriction of pHSV-106 correspond to the pBR-322 component (4.3 kb) and the HSV-tk component (3.4 kb) of the recombinant. Lanes 3-10 of the gel shown on the left of Figure 5a were loaded with a Hind III/Bam HI double digest of the plasmid DNA of one of eight 5' deletion mutants. Lanes 3-9 of the gel shown on the right were loaded with Hind III/Bam HI digests of seven 3' mutant isolates. B) Schematic diagram of the dimensions of each deletion mutant with respect to a restriction enzyme map of the 3.4 kb tk insert in pHSV-106. The solid lines above the restriction enzyme map diagram the DNA sequences remaining in each 5' deletion mutant. The lines below the restriction map show the sequences remaining within each 3' deletion mutant. The nomenclature of the deletion mutants is derived from the amount of DNA in kilobases that is missing from either the leftward Bam HI site of the parental plasmid (5' deletion mutants, Δ5') or from the rightward Bam HI site of pHSV-106 (3' deletion mutants, Δ3').

derived from the position of the end point of the deletion as determined by restriction enzyme mapping. 5' deletion mutants are designated by the prefix Δ5'. The numerology following the prefix refers to the amount of DNA, in kb, deleted from the tk component of the parental plasmid. For example, the isolate Δ5'-0.20 was determined by restriction analysis to have lost approximately 0.2 kb (200 bp) from the leftward Bam HI site of pHSV-106. Similarly, Δ3'-1.94 is missing 1.94 kb (1,940 bp) from the rightward Bam HI site of pHSV-106.

Analysis of tk Deletion Mutants by Oocyte Injection:

Each deletion mutant described in the preceding section was tested for its ability to support tk synthesis when microinjected into the nuclei of frog oocytes. For each assay, approximately 5 ng of closed circular plasmid DNA was injected into each of 50 oocytes. Following a 24 hour incubation interval the surviving oocytes were homogenized and assayed for tk enzymatic activity as descrived in Materials and Methods. Results of the assays are presented in Table IV. The first deletion toward the 3' end of the tk gene that alters its expression is Δ3'-1.60. The largest 3' deletion that does not affect the gene is Δ3'-1.32. Thus, the oocyte assays on 3' deletion mutants delimit a 3' functional boundary of the HSV tk gene to the region separated by the end points of Δ3'-1.60 and Δ3'-1.32.

Three of the eight 5' deletion mutants support tk synthesis in microinjected oocytes (Table IV). Of the three, only the smallest deletion, Δ5'-0.20, directs a significant increase in tk enzymatic activity in frog oocytes. Δ5'-0.67 and Δ5'-0.72 reproducibly stimulate tk synthesis in oocytes, but to a markedly reduced level as compared with the parental plasmid or Δ5'-0.20. These results delimit a 5' functional boundary of the HSV tk gene to the sequences between the end points of deletion mutants Δ5'-0.20 and Δ5'-1.01. The reduced functional capacities of Δ5'-0.67 and Δ5'-0.72 suggest that the end points of these mutants may lie close to the 5' terminus of the gene.

The results of the experiments presented in Table IV suggest that the functional mutants of the series of 3' deletions stimulate tk enzyme synthesis to only 1/3 the level of the single, fully functional 5' deletion mutant (Δ5'-0.20). This result is due to the fact that the two series of mutants were assayed using the oocytes of different female frogs. When the functional 3' deletions are assayed in concert with Δ5'-0.20 and the parental recombinant (pHSV-106) on oocytes from a singly female, similar levels of enzyme induction are observed (data not shown).

TABLE IV. OOCYTE INJECTION ASSAYS OF TK DELETION MUTANTS

Deletion isolate	Stimulation in tk activity above oocyte background
Δ5'-0.20	224X
Δ5'-0.67	12.9X
Δ5'-0.72	3.3X
Δ5'-1.01	nd
Δ5'-1.09	nd
Δ5'-1.19	nd
Δ5'-1.24	nd
Δ5'-1.34	nd
Δ3'-0.21	63X
Δ3'-0.57	86X
Δ3'-0.77	71X
Δ3'-1.13	60X
Δ3'-1.32	78X
Δ3'-1.60	nd
Δ3'-1.94	nd

Injection assays of all 5' deletion mutants were performed on oocytes dissected from the same frog. For each deletion isolate, 50 oocytes were injected with 10 ng of supercoiled plasmid DNA. After 24 hours incubation at room temperature oocytes were homogenized and assayed for tk enzyme activity as described in Materials and Methods. The same protocol was used for assaying the series of 3' deletion mutants. All 3' deletion mutants were injected into oocytes prepared from the same frog (yet different from the one used to assay the 5' deletion mutants). Results of oocyte injection assays are expressed as the degree of stimulation in tk activity above that of uninjected oocytes. nd refers to samples in which no detectable increase in tk enzyme activity was observed.

DISCUSSION

We have presented evidence that upon microinjection in *Xenopus laevis* oocytes the HSV thymidine kinase gene can be expressed in the form of functional tk enzyme. We have confirmed the validity of this observation in three ways: first, the tk enzyme synthesized in oocytes in response to the injection of HSV tk DNA is inactivated specifically by anti-tk IgG; second, the anti-tk IgG precipitates a polypeptide unique to tk-injected oocytes that has an apparent molecular weight equivalent to the tk protein synthesized in HSV infected cells; third, the tk enzymatic activity synthesized in response to injected tk DNA can utilize deoxycytidine as a substrate. It is known that microinjected oocytes are capable of expressing structural genes (DeRobertis and Mertz, 1977; Rungger and Turner, 1978; Probst *et al.*, 1979). The present study, however, is the first example of the expression of an active enzyme by its gene in the coupled

transcription/translation system of the oocyte.

A second objective of these experiments was to locate the tk gene within the 3.4 kb Bam HI fragment of HSV I. It is known that the HSV tk polypeptide migrates at an apparent molecular weight of 42,000 daltons on SDS polyacrylamide gels (Summers et al., 1975; Cremer et al., 1978). The mRNA for a protein of this size requires at least 1,100 ribonucleotides. Since the HSV-tk Bam HI fragment cloned in pHSV-106 measures roughly 3.4 kb, we reasoned that the functional boundaries of the tk gene might be narrowed down significantly. One available approach to this objective was outlined by Wigler et al. (1977) in their original report on DNA mediated cell transfection. They cleaved the 3.4 kb Bam HI fragment with a variety of restriction enzymes and showed that some enzymes that cleave the 3.4 kb Bam HI fragment render it incapable of transfecting tk^- cells to the tk^+ genotype. It was concluded that these enzymes cleave the DNA sufficiently near to the tk gene to destroy its capacity to function in recipient cells. In a series of more recent reports on the HSV tk gene, this restriction mapping approach has been extended using cloned fragments of HSV tk DNA (Colbere-Garapin et al., 1979; Wilkie et al., 1979; Enquist et al., 1980). Colbere-Garapin et al. (1979) found that digestion of tk DNA with the enzyme PvuII does not inactivate the tk gene. The same study also showed that SmaI restriction of tk DNA results in a 90-95% inactivation in the transfection assay. These results indicate that the functional tk gene residues fully within the 2.03 kb Pvu II fragment of the tk isolate used in their study, and that a Sma I restriction enzyme recognition site may be located adjacent to the 3' terminus of the tk gene.

We chose a different approach to map the functional extent of the HSV tk gene. In order to eliminate a dependence upon naturally occurring restriction enzyme sites, we constructed two sets of deletion mutants of the HSV tk gene. One set of mutants is deficient in varying amounts of the 5' flanking and N-terminus specific sequences of the tk gene. The other set is deficient in 3' flanking and carboxyl-specific sequences. By testing the functional capacity of each deletion mutant in the oocyte assay system, we have delimited the boundaries of the DNA required for functional expression of the tk gene. We predict that the functional tk gene resides between the end points of deletion mutants Δ5'-0.20 and Δ3'-1.32. These deletion mutants and all isolates retaining greater extents of the tk DNA fragment cloned in pHSV-106, function adequately in the oocyte injection assay. We have also found that two 5' deletion mutants, having end points 670 and 720 bp from the leftward Bam HI site of the parental 3.4 kb Bam HI fragment, elicit reduced

levels of tk enzyme synthesis in oocytes. It should be pointed out that both of these 5' deletion mutants (Δ5'-0.67 and Δ5'-0.72) remove the leftward Eco RI site of the tk DNA of pHSV-106. Wigler et al. (1977) reported that Eco RI digestion of the 3.4 kb tk fragment of HSV I inactivates the DNA in a transfection assay. Thus, it appears that the DNA in a region extending from the Eco RI site to the end point of Δ5'-0.72 (110 bp) is required for the gene to be expressed maximally. The end points of Δ5'-0.67 and Δ5'-0.72 may be loacted in sequences flanking the 5' terminus of the tk gene or, possibly, in a portion of the gene complementary to the 5' untranslated segment of tk mRNA. The observation that both Δ5'-0.67 and Δ5'-0.72 support the synthesis of detectable levels of tk enzymatic activity in oocytes, however, suggests that the entire protein coding component of the tk gene is retained by both mutants.

Finally, we point out that the combined data of the present study and that of Colbere-Garapin et al. (1979) delimit the tk gene to a segment of DNA no greater than 1,550 bp (Pvu II site to Δ3'-1.32), and possibly as small as 1,190 bp (Δ5'-0.72 to the Sma I site). These observations suggest that the tk gene is not appreciably larger than the minimum size required to code for the tk polypeptide. Thus, the tk gene is probably not interrupted by intervening DNA sequences. In the accompanying report, physical evidence supporting this conclusion is presented (McKnight, 1980).

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