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mRNA- and DNA-Directed Synthesis of Herpes Simplex Virus-Coded Exonuclease in *Xenopus laevis* Oocytes

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Microinjection of herpes simplex virus (HSV)-infected cell mRNA into *Xenopus laevis* oocytes resulted in the production of a new exonuclease activity. This enzyme strongly resembled the HSV alkaline exonuclease in many biochemical properties, and hybrid-arrested translation studies showed that it was virus coded, mapping at 0.080 to 0.185 genome map units. Exonuclease mRNA had a size and genome location equivalent to the mRNA encoding VI85 in reticulocyte lysates, suggesting that VI85 is the exonuclease. The enzyme synthesized in oocytes was found to act as an exonuclease in vivo. Two plasmids containing HSV DNA fragments directed the synthesis of exonuclease when microinjected into oocyte nuclei, and this finding enabled the coding and control sequences for this gene to be localized to 0.155 to 0.185 genome map units.

Infection of cells with herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) results in the production of an alkaline exonuclease (9, 10, 21, 28). At least one essential component of this enzyme is virus coded, since the activity induced by the HSV-2 mutant *ts*13 is thermolabile (7, 23). The role of the exonuclease in the virus replication cycle is unclear at present.

It has been shown previously that mRNA-directed synthesis of HSV-specified thymidine (pyrimidine deoxyribonucleoside) kinase (TK) can be detected in the reticulocyte lysate cell-free system, provided appropriate assay conditions are used (5, 25, 29). These methods have facilitated studies on the size and genome map location of TK mRNA (5, 27), and have enabled the transcriptional control of the TK gene to be investigated (26). We report here that microinjection of HSV-infected cell mRNA into *Xenopus laevis* oocytes results in the production of active exonuclease, and therefore that similar studies are possible with this enzyme. We further show that synthesis of HSV exonuclease occurs after microinjection of suitable plasmid DNAs into oocyte nuclei.

MATERIALS AND METHODS

Plasmid DNAs. The plasmid DNAs used in these studies were as follows: (i) pGX41, which consists of the HSV-1 *Eco*RI D fragment cloned into the *Eco*RI site of pACYC184 (prepared by B. Matz); (ii) pGX24, which consists of the HSV-1 *Bam*HI A fragment cloned into the *Bam*HI site of pAT153 (prepared by V. Preston); and (iii) pGZ65, which consists of the HSV-2 *Bam*HI F fragment cloned into the *Bam*HI site of pAT153 (prepared by A. Davison). For microinjection into *X. laevis* oocytes, plasmid DNAs were purified by sucrose density gradient centrifugation.

RNA preparations. Total cytoplasmic RNA was extracted from BHK cells at 5 h after infection with 20 PFU of HSV-1 or HSV-2 per cell at 31°C, as described previously (24). Polyadenylated RNA [poly(A)+ RNA] was selected from total cytoplasmic RNA by oligodeoxythymidylic acid-cellulose chromatography.

For size fractionation, RNA was denatured by incubation in 50% formamide–50 mM PIPES [piperazine-*N*-*N*'-bis(2-ethanesulfonic acid)] buffer (pH 7.4) at 60°C for 10 min, diluted twofold, and loaded onto a 13-ml 15 to 40% sucrose density gradient prepared in 10 mM Tris-hydrochloride–10 mM NaCl–1 mM EDTA, pH 7.5. Centrifugation was at 40,000 rpm in a Beckman SW40 Ti rotor for 16 h at 15°C. Gradients were fractionated, and NaCl was added to individual fractions to a final concentration of 200 mM. RNA was precipitated by the addition of two volumes of ethanol, and the precipitates were washed with ethanol, dried, and dissolved in deionized water.

Hybrid-arrested translation with plasmid DNAs was performed as described previously (6, 27).

Microinjection of RNA and DNA into *X. laevis* oocytes. Female *X. laevis* were purchased from Xenopus Ltd., Redhill, England. Stage 5 oocytes were microinjected by the use of a glass micropipette, with 50 nl of RNA solution [5 mg/ml for total cytoplasmic RNA or 150 μg/ml for poly(A)+ RNA] aiming at the cytoplasm, or 20 nl of plasmid DNA solution (200 μg/ml) aiming at the nucleus as described by Gurdon (8). Groups of five oocytes were incubated in modified Barth medium (8) at 22°C for 40 to 48 h. They were then manually disrupted and prepared for enzyme assays as described by Cordingley and Preston (3).

Enzyme assays. TK was assayed by following the phosphorylation of [*methyl*-^{3}H]thymidine, as described previously (13, 25). TTP was added at 50 μM to suppress endogenous reticulocyte or oocyte enzymes (3, 25).

The substrate used for exonuclease assays was *Bam*HI-cleaved pGZ65 DNA (this plasmid was chosen arbitrarily). This substrate was incubated with DNA

polymerase large fragment (Bethesda Research Laboratories, Gaithersburg, Md.) and unlabeled dATP, dCTP, and TTP (100 μM each) plus [α-^{32}P]dGTP (The Radiochemical Centre, Amersham, England; specific activity, 2,000 to 3,000 Ci/mmol), using the assay conditions recommended by the supplier. After 2 h at 15°C, the reaction mixture was extracted once with phenol-chloroform (1:1, vol/vol) and applied to a Sephadex G-50 column. The excluded fractions were collected and precipitated with ethanol. The final specific activity of the substrate was approximately 1 × 10^6 to 2 × 10^6 cpm (Cerenkov) per μg.

Exonuclease assays contained 50 mM Tris-hydrochloride (pH 8.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl, approximately 10^5 cpm of 3'-end-labeled [^{32}P]DNA, and 5 μl of oocyte extract in a final volume of 25 μl. Reactions were incubated at 30°C for 1 h, after which 25 μl of 20% trichloroacetic acid was added. Samples were kept on ice for 30 min, then centrifuged at 13,000 × *g* for 2 min. The supernatant was carefully removed, and released Cerenkov radioactivity was measured. The radioactivity in the pellet was also determined. A zero-time control was always performed to correct for any acid-soluble radioactive material in the substrate. Exonuclease activity was expressed as the percentage of initial radioactivity rendered acid-soluble (see Fig. 1, 3 and 5), or the Cerenkov counts per minute released from an initial 10^5 cpm of substrate (see Tables 1 to 3).

Intracellular degradation of DNA in oocytes. End-labeled DNA, prepared as described above, was microinjected into the cytoplasm of oocytes which had been injected with infected BHK cell mRNA 40 h earlier. At various times, oocytes were manually disrupted in 20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 200 μg of proteinase K per ml, and incubated at 37°C for 2 h. Samples were then extracted twice with phenol-chloroform and once with chloroform. Cerenkov radioactivity was measured in the final aqueous phase, and samples containing equal radioactivity were loaded onto a 0.5% agarose gel (2). The gel was run overnight at 40 mA, dried, and exposed to Kodak XH-1 film for autoradiography.

In vitro protein synthesis. mRNA was translated in a fractionated reticulocyte cell-free system, and radiolabeled polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (24, 26).

RESULTS

In vitro synthesis of HSV exonuclease. Previous studies have shown that mRNA-directed synthesis of HSV TK in reticulocyte lysates can be detected, provided specific and sensitive assay methods are used (3, 5, 20, 25). To modify the exonuclease assay for increased sensitivity, *Bam*HI-cleaved DNA radiolabeled to high specific activity by various methods was tested as substrate, and the most effective approach was found to be 3'-end-labeling with [α-^{32}P]dGTP. Exonuclease activity was therefore measured by removal of the terminal radioactive nucleotides. Samples of RNA preparations from infected or uninfected BHK cells were either translated in the reticulocyte lysate system or microinjected into *X. laevis* oocytes. After suitable incubation periods, extracts were analyzed for TK and exonuclease activities (Table 1). HSV TK and a new nuclease could be detected in both systems, but clearly the nuclease was synthesized more efficiently, using TK as a standard, in oocytes than in reticulocytes. The possibility that enzyme assays were affected by inhibitory components in the reticulocyte lysate or stimulatory factors in the oocyte extracts was eliminated by performing appropriate mixing experiments. Microinjection into *X. laevis* oocytes is therefore the system of choice for mRNA-directed synthesis of exonuclease.

In this and subsequent experiments, mock-infected BHK cell RNA was microinjected at the same concentration as infected cell RNA to provide an appropriate control. The background activity, which was due to an oocyte nuclease, was unaffected by microinjection of the following materials: water, *Escherichia coli* rRNA, vesicular stomatitis virus-infected or mock-infected BHK cell total cytoplasmic RNA at concentrations up to 5 mg/ml, or BHK cell poly(A)+ at concentrations up to 500 μg/ml. Therefore, no evidence was obtained for synthesis of a BHK cell nuclease or for stimulation of oocyte nucleases by the microinjection procedure.

Quantitation of exonuclease synthesized in oocytes. The assay method for the detection of exonuclease measures removal of only the terminal nucleotides from radiolabeled DNA; therefore the specific activity of the substrate at its 3'-termini should fall during the reaction, causing an apparent decrease in rate. The same

TABLE 1. Synthesis of HSV enzymes in reticulocyte lysates and *X. laevis* oocytes

Extract	Exonuclease[a] (cpm)	TK (cpm)
Reticulocyte (INF RNA)[b]	6,636	39,994
Reticulocyte (MI RNA)	1,934	2,246
Reticulocyte (INF RNA) plus oocyte (MI RNA)[c]	7,451	38,211
Oocyte (INF RNA)	50,726	31,702
Oocyte (MI RNA)	2,876	3,219
Oocyte (INF RNA) plus reticulocyte (MI RNA)	36,662	18,292

[a] Exonuclease assays were performed at pH 9.5 instead of pH 8.5.

[b] Reticulocyte lysates were incubated with HSV-1-infected (INF) or mock-infected (MI) BHK cell RNA.

[c] Oocytes were microinjected with HSV-1-infected (INF) or mock-infected (MI) BHK cell RNA, and extracts were made 40 h after microinjection.

effect would result from any endonuclease activity, since unlabeled ends would be generated. The response of the exonuclease assay to alterations in the concentration of oocyte extract or incubation time was therefore investigated.

The results in Fig. 1A show that enzyme activity was proportional to extract concentration until approximately 50% of the initial radioactivity was rendered acid soluble, after which little change was observed. A time course of the reaction (Fig. 1B) showed that release of nucleotides was proportional to incubation time until 50% of the initial radioactivity was acid soluble. When 2 instead of 5 μl of extract was added, the initial rate was correspondingly lower, but release of nucleotides continued longer, showing that the enzyme was relatively stable during the reaction. The assay conditions described here therefore give a quantitative estimate of exonuclease activity, provided no more than 50% of the initial radioactivity is released from the substrate. In all subsequent experiments, at least two different extract concentrations were used, and any experimental points which represented greater than 50% release of the initial radioactivity were discarded.

Figure 2 shows that exonuclease activity was proportional to the concentration of poly(A)+ RNA microinjected into oocytes up to 150 μg/ml, above which the rise in enzyme level was more gradual.

Characterization of exonuclease synthesized in oocytes. The new enzyme synthesized in oocytes after microinjection of infected cell mRNA was found to be similar to the major exonuclease in HSV-infected BHK cell extracts in the following properties. (i) Both activities were resistant to inhibition by 100 μg of phosphonoacetic acid per ml or 0.1 mM $ZnSO_4$, distinguishing them from the DNA polymerase-associated exonuclease activity (15). (ii) Addition of 1 mM $CaCl_2$ inhibited both activities (28) but stimulated uninfected BHK cell and oocyte nucleases. (iii) The two enzymes bound to DEAE-cellulose at low salt and pH 8.5 and were eluted at 0.20 to 0.25 M NaCl (21). (iv) The pH optimum for both activities was in the range pH 8.5 to 9.5.

It has been shown that the HSV-2 mutant *ts*13

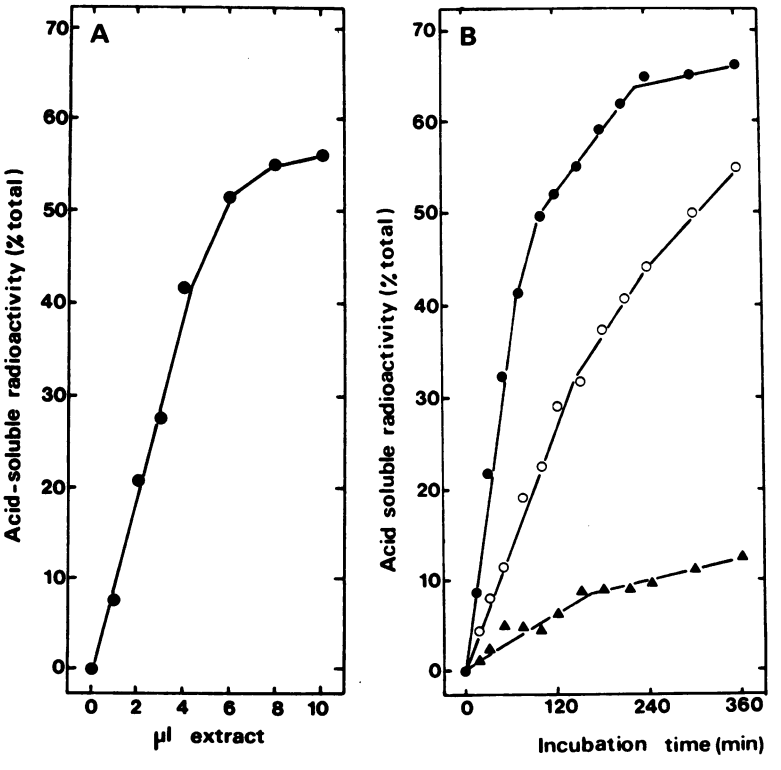

FIG. 1. Quantitation of exonuclease synthesis. Oocytes were microinjected with HSV-1-infected or mock-infected BHK cell RNA, and extracts were assayed for exonuclease. (A) Various amounts of extract from infected cell RNA-injected oocytes were added to a 25-μl reaction mixture. Incubation was for 2 h. (B) Undiluted (●) or a two-fifths dilution of infected cell RNA-injected extract (○), or mock-infected cell RNA-injected extract (▲) was incubated for various times.

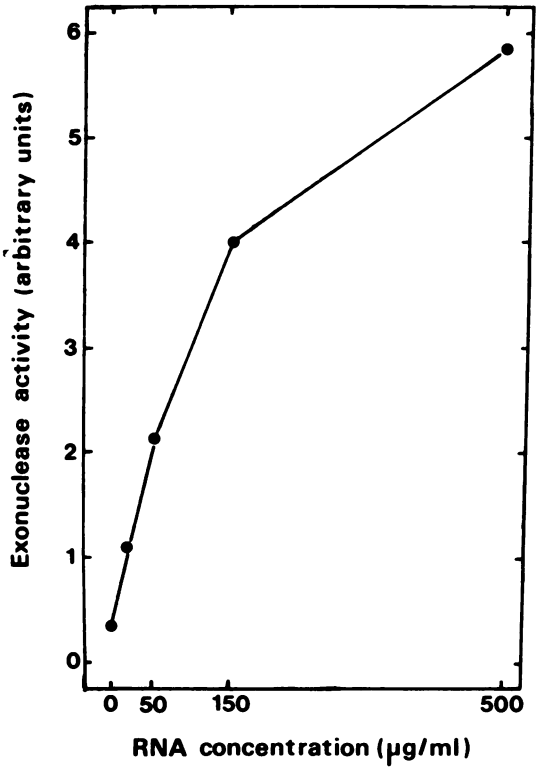

FIG. 2. Relationship between exonuclease synthesis and mRNA concentration. Oocytes were microinjected with infected BHK cell poly(A)+ RNA at various concentrations. For each point, various extract concentrations were tested, and only those giving less than 50% of radioactivity released were used to calculate exonuclease activity. The arbitrary units are a function of exonuclease activity and the dilution factor for each oocyte extract.

induces a thermolabile exonuclease in BHK cells (7). The inactivation at 45°C of nucleases in oocytes after microinjection of RNA from BHK cells infected with wild-type HSV-2 or *ts*13 was therefore examined (Fig. 3). The activity induced by *ts*13 RNA was more labile than that induced by wild-type HSV-2 RNA, the times for 50% inactivation being 2.8 and 5.1 min, respectively. It should be noted that the endogenous oocyte enzyme was more than 90% inactivated after 1 min at 45°C and is therefore much more thermolabile than the induced activity. This experiment strongly suggests that the new enzyme synthesized in *X. laevis* oocytes is authentic HSV exonuclease.

The mRNA-directed exonuclease was further examined by hybrid-arrested translation. It has previously been shown that the enzyme maps at the genome position 0.12 to 0.21 (23), and therefore plasmid pGX41, which contains the HSV-1 DNA fragment *Eco*RI D (map coordinates 0.080 to 0.185) was chosen for hybridization to infected cell mRNA. The mRNA was then microinjected into oocytes either directly or after denaturation of hybrids, and exonuclease activities were analyzed after incubation for 40 h. TK activity was also measured to provide a control. The results in Table 2 show that hybridization with pGX41 DNA specifically inactivated exonuclease mRNA and that its activity returned, to some extent, after denaturation of hybrids. Incubation of mRNA with pACYC184 DNA also caused a decrease in activity of this mRNA, suggesting that it may show the "self-arrest" property described previously for some HSV mRNAs (6, 27).

The RNA preparations used in this experiment were also analyzed by translation in reticulocyte lysates and subsequent separation of polypeptides by sodium dodecyl sulfate-poly-

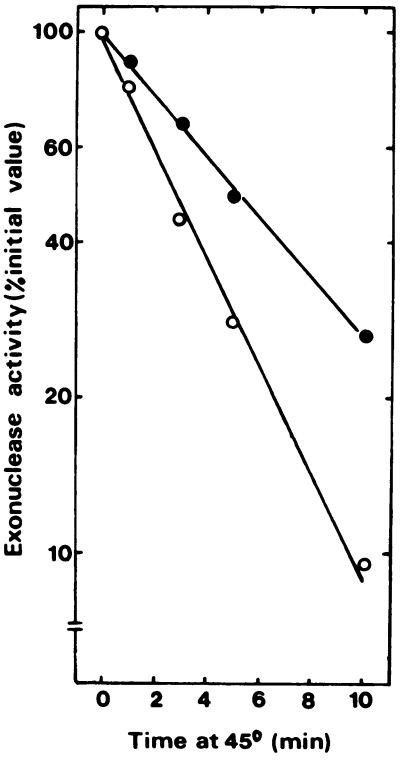

FIG. 3. Thermal inactivation of exonucleases synthesized in oocytes. RNA from cells infected with wild-type HSV-2 (●) or *ts*13 (○) was microinjected into oocytes. The resulting oocyte extracts had equivalent exonuclease and TK activities when assayed at 30°C and had equivalent protein contents. For thermal inactivation, extracts were preincubated for various times at 45°C before assay at 30°C. Unlabeled substrate DNA (0.1 µg) was added to assay mixtures before preincubation. Values from uninjected oocyte extracts were subtracted from all values.

TABLE 2. Hybrid-arrested translation of exonuclease mRNA[a]

RNA	Exonuclease (cpm)	TK (cpm)
pGX41 hybridized	2,067	24,805
pGX41 hybridized and denatured	15,459	42,701
pACYC184 hybridized	11,452	19,474
pACYC184 hybridized and denatured	34,613	43,146
BHK cell	1,242	3,290

[a] Infected cell RNA was hybridized to plasmid DNAs and microinjected into oocytes either directly or after denaturation. Extracts were made after incubation at 22°C for 40 h.

acrylamide gel electrophoresis (Fig. 4). It can be seen that synthesis of polypeptide VI85 was totally arrested by hybridization with pGX41 DNA (Fig. 4, track 4), and it returned, although not completely, when the RNA preparation was denatured before translation (Fig. 4, track 5). No other *Eco*RI D-specific polypeptides were detected by hybrid-arrested translation, possibly reflecting the absence of major mRNAs homologous to this region in the "early" RNA preparation used (11).

Hybrid-arrested translation with the cloned HSV-1 *Bam*HI P fragment has been described previously (27). It was found, (Fig. 4, tracks 6 and 7) that some mRNAs are totally (VI136 and VI88) or partially (VI85) inactivated by mock hybridization with an unrelated DNA or without DNA. The partial inactivation of VI85 mRNA by pACYC184 DNA or cloned *Bam* HI P fragment DNA (27) contrasts with the total inactivation by *Eco*RI D DNA (Fig. 4, track 4). The previous studies (27) suggested that VI61 was either a *Bam* HI P-specified polypeptide or a self-arresting band, and the results shown in Fig. 4 confirm the latter to be the case.

The hybrid-arrested translation experiments therefore confirm that the new exonuclease synthesized in oocytes is HSV specified and that it maps in the expected genome region. The species VI85 is a candidate for the corresponding polypeptide.

The exonuclease was examined further by fractionating infected-cell RNA on a sucrose density gradient. Individual fractions were recovered and analyzed either by microinjection into *X. laevis* oocytes, for production of TK and exonuclease, or by translation in reticulocyte lysates for the detection of polypeptides (Fig. 5). The results show that exonuclease mRNA sedimented more rapidly than TK mRNA, which showed the expected size of approximately 16S (5). Analysis of the polypeptides synthesized in reticulocyte lysates revealed that the mRNA encoding VI85 sedimented in the same fractions as exonuclease mRNA, giving further evidence for a relationship between VI85 and the enzyme.

The sizes of specific mRNAs for some of the polypeptides synthesized in vitro have been determined by electrophoresis in denaturing gels (1, 4; J. McLauchlan and J. B. Clements, submitted for publication), and these were used in an attempt to calibrate the sucrose density gradient. An estimated size of 3.2 ± 0.5 kilobases was calculated for exonuclease mRNA.

It should be noted that the sedimentation rate of most mRNAs is related to the size of the polypeptide product. There are cases, however, in which the mRNA sediments considerably more rapidly than expected from the size of its

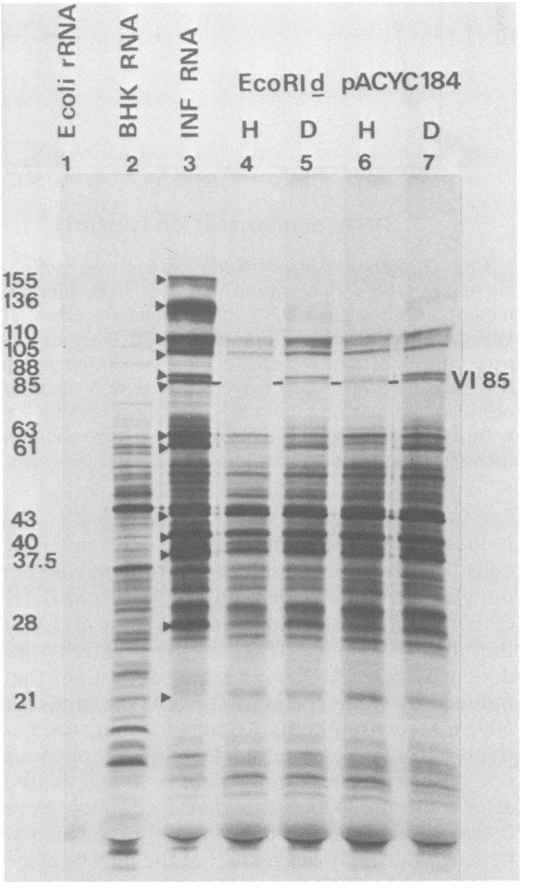

FIG. 4. Hybrid-arrested translation of exonuclease mRNA. Infected cell RNA was hybridized to pGX41 (tracks 4 and 5) or pAT153 (tracks 6 and 7) DNA and translated directly (H) or after denaturation (D). Also shown are translation products of infected cell RNA (track 3), BHK cell RNA (track 2), or *E. coli* rRNA (track 1). Polypeptides synthesized in reticulocyte lysates are classified as described previously (24).

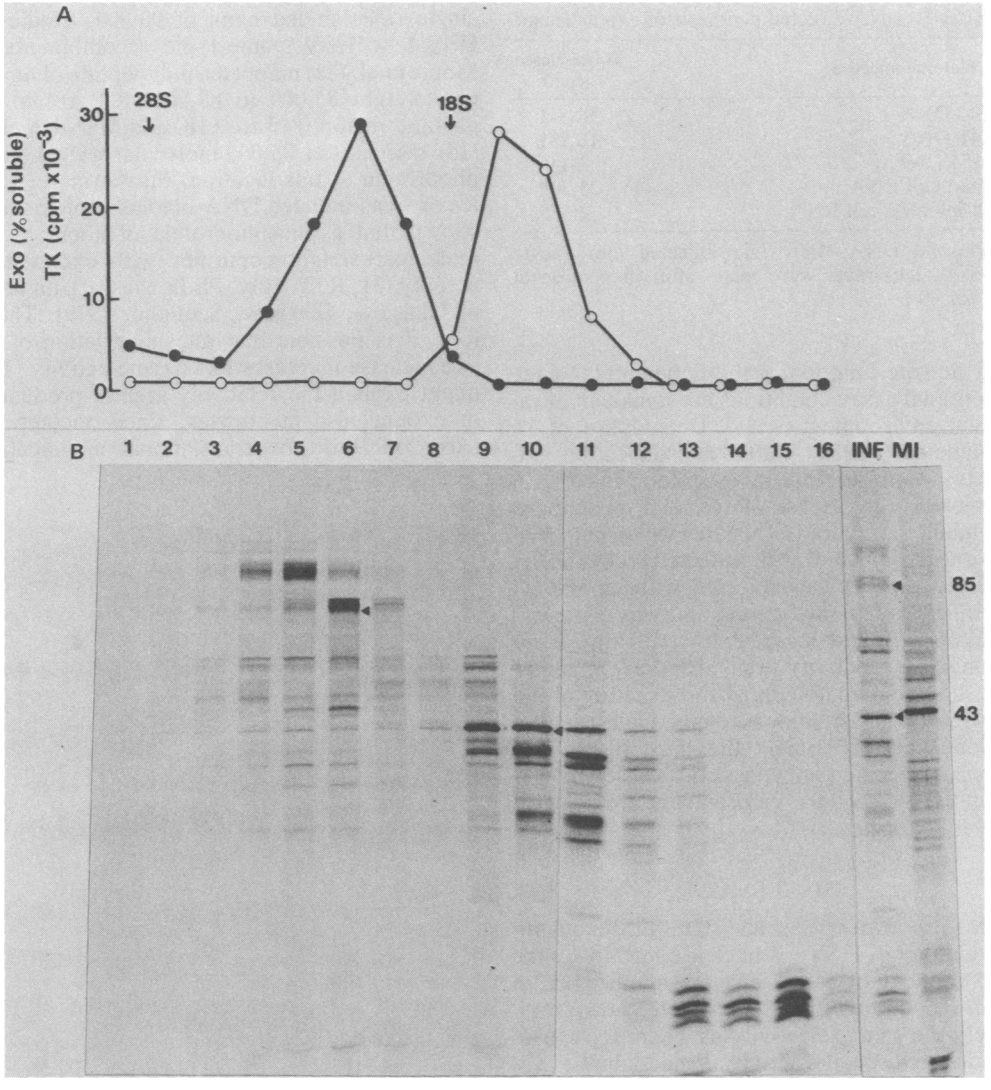

FIG. 5. Size fractionation of mRNAs. Infected cell mRNAs were separated on a sucrose density gradient, and fractions were analyzed for (A) synthesis of exonuclease (●) and TK (○) in oocytes, or (B) synthesis of radiolabeled polypeptides in reticulocyte lysates.

encoded products, and others in which polypeptides of equal apparent molecular weights are synthesized from mRNAs of different size classes. The significance of these observations is unclear at present.

DNA-directed synthesis of HSV exonuclease. Previous studies have demonstrated that plasmid DNAs containing the HSV TK gene can direct the synthesis of active enzyme when microinjected into nuclei of *X. laevis* oocytes (3, 19, 20). Plasmids containing HSV DNA fragments mapping in the exonuclease region were microinjected into oocyte nuclei, and after incubation for 48 h, extracts were assayed for enzyme activity. It was found that plasmid pGX41, which contains *Eco*RI fragment D (0.080 to 0.185 genome map units) or pGX24, which contains *Bam*HI fragment A (0.145 to 0.225) resulted in the synthesis of functional exonuclease (Table 3). This enzyme had an equivalent pH optimum and sensitivity to $CaCl_2$ inhibition to the mRNA-directed activity (data not shown). The genome location 0.145 to 0.185 (approximately 7 kilobases) therefore contains all the coding and noncoding sequences necessary for expression of the HSV exonuclease gene in oocytes.

Intracellular activity of HSV exonuclease. It has been speculated that the exonuclease activity found in HSV-infected cells may not repre-

TABLE 3. DNA-directed synthesis of exonuclease[a]

Material injected	Exonuclease (cpm)
pGX24 DNA	16,541
pGX41 DNA	18,781
pAT153 DNA	2,145
Infected cell RNA	23,303
Mock-infected cell RNA	2,175

[a] Plasmid DNAs were microinjected into oocyte nuclei, and extracts were made after incubation at 22°C for 40 h.

sent the true function of the polypeptide, since the optimal assay conditions are unlike an intracellular environment (9, 28). Degradation of 3'-end-labeled [^{32}P]DNA after microinjection into oocytes was therefore investigated, and Fig. 6 shows that the release of terminal nucleotides was much more rapid in oocytes which had been previously injected with infected cell mRNA than those previously injected with uninfected cell mRNA. The rate of reaction was comparable in oocytes and oocyte extracts. Although no endonuclease activity was observed in either case, it should be noted that the structure of the substrate would militate strongly against its detection. This experiment therefore shows that the virus-specified enzyme acts as an exonuclease in the intracellular environment provided by *X. laevis* oocytes.

DISCUSSION

This report describes a system for the quantitative assay of HSV exonuclease mRNA by the measurement of active enzyme synthesized in *X. laevis* oocytes. It can be detected at approximately the same sensitivity as TK mRNA, provided a high specific activity 3'-end-labeled substrate is used. An oocyte nuclease gives only a low background activity, and this can effectively be abolished by preincubation of samples at 45°C for 1 min before assay. The mRNA-directed exonuclease has biochemical properties indistinguishable from the enzyme synthesized in BHK cells, suggesting that any cellular components necessary for its activity can also be supplied by oocytes.

The studies presented here suggest that VI85, synthesized in reticulocyte lysates, is the exonuclease polypeptide since it has the correct genome location and mRNA size. This conclusion is in good agreement with previous findings, particularly the work of Strobel-Fidler and Francke (28), in which purified exonuclease from [^{35}S]methionine-labeled infected cells was examined. The enzyme resolved into three polypeptides of molecular weights 90,000, 85,000, and 70,000 by analysis of total protein but gave a single radiolabeled band of 90,000. Studies of HSV-1 × HSV-2 intertypic recombinants by Morse et al. (22) mapped a polypeptide of molecular weight 85,000 to 88,000 (ICP 18) to the genome region 0.15 to 0.18, and Marsden et al. (18) mapped an 85,000-molecular-weight phosphoprotein at this location. Furthermore, studies of virus-induced DNA-binding proteins have shown that a phosphoprotein of about 85,000 molecular weight copurifies with exonuclease activity (31; R. T. Hay, Ph.D. thesis, University of Glasgow, Glasgow, Scotland, 1979). Therefore, it is possible that phosphorylation of the exonuclease increases its enzyme activity. This might explain the relatively greater production of exonuclease in oocytes, since oocytes can carry out this post-translational modification,

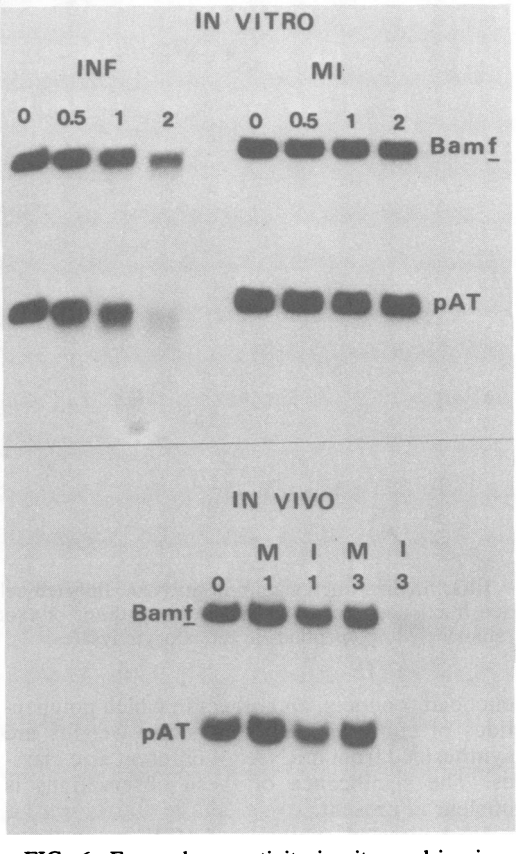

FIG. 6. Exonuclease activity in vitro and in vivo. (A) Oocytes were microinjected with infected (INF) or mock-infected (MI) cell RNA, and extracts were assayed for degradation of radioactive substrate after 0, 0.5, 1, or 2 h of incubation at 30°C. (B) Radiolabeled DNA was microinjected into oocytes which had previously been injected with infected (I) or mock-infected (M) cell RNA. Nucleic acids were extracted at 0, 1, or 3 h after injection of DNA and assayed for degradation of radioactive substrate.

whereas HSV phosphoproteins synthesized in reticulocyte lysates migrate on polyacrylamide gels as unmodified precursors (16, 24, 30; and C. Preston, unpublished data).

Microinjection of plasmid DNAs has shown that the coding sequences and any control sequences necessary for expression of the exonuclease in oocytes map in the genome region 0.145 to 0.185, which is approximately double the requirement for a 3.2-kilobase mRNA. This finding will allow the sequences important for transcription to be mapped and manipulated in the way used for TK (19, 20). It is clear, however, that HSV exonuclease, like TK, is a gene which normally requires an active immediate-early polypeptide (Vmw175) for its expression in infected cells but not in *X. laevis* oocytes (12, 26). At present, it is unknown whether the amounts of these enzymes produced in oocytes are due to a basal level of transcription which could be amplified by correct interaction with Vmw175 as found in TK-transformed L cells (14, 17), or if they represent a high level of gene expression due to the absence of modulating factors normally present in infected cells. In either case, the system described here should be useful in a search for viral and cellular polypeptides which affect transcription of the HSV exonuclease gene.

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