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Biochemistry

Casein kinase II specifically nucleotidylylates *in vitro* the amino acid sequence of the protein encoded by the α22 gene of herpes simplex virus 1

(adenylylation/guanylylation/regulation)

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ABSTRACT **An earlier report has shown that eight viral proteins with a common amino acid sequence (R/P)RA(P/S)R are nucleotidylyated *in vitro* by nuclear extracts from cells infected with herpes simplex virus 1. One, the product of the α22 gene, is nucleotidylylated in the absence of viral proteins made late in infection. A chimeric protein (GST22P) consisting of amino acids 50–200 of the α22 coding sequence fused to the C terminus of the glutathione *S*-transferase was nucleotidylylated by enzymes in nuclear extracts of infected or mock-infected cells and also by a casein kinase II enzyme purified from the sea star. The enzyme did not nucleotidylylate common casein kinase II substrates (casein, phosvitin) and the reaction was inhibited by heparin. The results are consistent with the hypothesis that nucleotidylylation of the eight viral proteins involves casein kinase II.**

The herpes simplex virus 1 (HSV-1) genome encodes at least 77 diverse open reading frames that express proteins (1–6). The genes studied in detail form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion at both the transcriptional and posttranscriptional levels (7–9). Four of the five α genes, the genes expressed immediately after infection, have regulatory functions and are required for the later expression of the β and γ groups. We have reported that these four α proteins, the infected cell proteins (ICPs) 0, 4, 22, and 27, the products of the α0, α4, α22, and α27 genes as well as the products of U_L21, U_L31, U_L47, and U_L49 genes of HSV-1 strain F [HSV-1(F)] can be labeled by [α-^{32}P]ATP, [α-^{32}P]GTP, and [2-^{3}H]ATP where the ^{3}H is in the adenine ring (Amersham). The labeling was predicted on the basis of the observation that the nucleotidylylated ICPs 22 and 27 share the amino acid sequence (R/P)RA(P/S)R and that the eight proteins are the only ones predicted to share this sequence. A combination of genetic and biochemical analyses independently verified the identity of the additional nucleotidylylated proteins (10, 11).

To identify the enzyme responsible for the labeling of these proteins, we cloned and expressed in *Escherichia coli* a chimeric gene (GST22P) consisting of the glutathione *S*-transferase (GST) fused in-frame with a portion of the open reading frame of the α22 gene. This substrate was labeled by [α-^{32}P]ATP, [α-^{32}P]GTP, and [2-^{3}H]ATP in the presence of nuclear extracts from uninfected cells. We also found that the nucleotidylylating activity copurified with a peptide identified as a subunit of the casein kinase II (CK-II) enzyme by its reactivity with antibody to the CKII protein and that the labeling activity in this extract was inhibited by heparin (J.A.B., C.M., L. McCormick, and B.R., unpublished data). Taken together, these observations suggested that CK-II might be involved in the labeling of GST22P.

CK-II is a serine (threonine) protein kinase present in all eukaryotes. The enzyme is usually present as a tetrameric protein complex consisting of either an $\alpha_2\beta_2$ and $\alpha\alpha'\beta_2$ structure. The CK-II used in our studies is derived from the sea star and is composed of one M_r 44,000 α subunit, one M_r 40,000 α′ subunit, and two M_r 28,000 β subunits (12). The α/α′ subunit has been shown to be capable of phosphotransferase activity as a monomer (13) but the addition of the β subunit stimulates phosphorylation of casein by >10-fold. The kinase activity is inhibited by heparin and is stimulated by basic substrates such as polylysine. Although autophosphorylation on the β subunits is common, the addition of basic substrates stimulates the autophosphorylation of the α subunit (14). The preferred site of phosphorylation seems to be a serine with an acidic or a phosphoserine residue in the third position N-terminal to the site of labeling (15–18).

We report that the sea star CK-II is capable of nucleotidylylating GST22P with [α-^{32}P]ATP, [α-^{32}P]GTP, and [2-^{3}H]ATP and that this labeling is inhibited by heparin.

MATERIALS AND METHODS

Cells and Viruses. HeLa S3 cells obtained from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (19). Subconfluent HeLa S3 cultures containing ≈4 × 10^6 cells were exposed to 5 plaque-forming units per cell for 1 hr and then incubated at 37°C in medium containing 2% calf serum.

Recombinant Plasmids. A small portion of the 5′ noncoding domain and the portion encoding the N-terminal domain of the ICP22 was cleaved with *Eco*NI and *Pvu* II from the *Bam*HI N fragment cloned as pRB138 (20); the ends were blunted with T4 DNA polymerase and inserted into the *Hin*cII site of the pGEM3Zf(+) vector (Promega) to create pRB4619. The α22 sequence coding for amino acids 50–200 was cloned from pRB4619 as a *Sty* I/*Hin*dIII fragment; the ends were blunted with T4 DNA polymerase and inserted into the *Bam*HI site of the pGEX2T vector (Pharmacia), which had been T4 blunted, generating pRB4654.

Production of GST22P. The plasmid (pRB4654) was transfected into *E. coli* BL21 cells and overnight cultures were grown in Luria broth (LB) containing 100 μg of ampicillin per ml. The overnight culture was diluted 1:10 into fresh LB

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Abbreviations: HSV-1, herpes simplex virus 1; ICP, infected cell protein; CK-II, casein kinase II; GST, glutathione *S*-transferase.

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containing 100 μg of ampicillin per ml and 0.0027% isopropyl β-D-thiogalactopyranoside and incubated at 37°C in a shaking incubator for 4 hr. The fusion protein, termed GST22P, was purified as recommended (21, 22).

RESULTS

The Chimeric Protein GST22P Is Nucleotidylylated by a Cellular Enzyme. Previously, we reported that in cells infected under conditions that restricted viral expression to α genes, only ICP22 was nucleotidylylated even though large amounts of other α proteins were made (10). To determine the origin of the enzyme, we constructed a chimeric protein by fusing amino acids 50–200 of ICP22 to the C terminus of GST. The GST22P produced in *E. coli* consisted of full-length as well as shorter degradation products. Fig. 1 shows that the chimeric protein was labeled *in vitro* with [α-^{32}P]ATP and enzymes contained in nuclear extracts of mock-infected or infected nuclear extracts. In this series of experiments, the nuclear extract was made from HeLa cells 20 hr after infection or mock infection, and the labeling protocol and the processing of the reaction mixture were done as described in the legend to Fig. 1. Reaction mixtures containing no fusion protein or GST served as controls. The salient features of the results were as follows:

(*i*) The GST22P proteins present in the reaction mixture consisted of full-length and truncated proteins (Fig. 1*A*). The full-length and several of the truncated GST22P proteins but not GST were labeled with [α-^{32}P]ATP by nuclear extracts from both infected and uninfected cells (Fig. 1*B*, lanes 1 and 4), whereas GST was not labeled under the same conditions (lanes 2 and 5). Neither protein was labeled in the absence of nuclear extracts (data not shown). Substitution of [α-^{32}P]ATP with [α-^{32}P]GTP yielded identical results (data not shown).

(*ii*) Labeling of the host proteins H2 and H3 identified previously (10) was abolished in the presence of GST22P.

We conclude that (*i*) the chimeric protein GST22P was nucleotidylylated and therefore could be used as substrate for *in vitro* analyses of nucleotidylylation, (*ii*) at least some of the truncated species of GST22P were also labeled but did not appear to interfere with the labeling of the full-length polypeptide, and (*iii*) the enzyme involved in the nucleotidylylation of GST22P with [α-^{32}P]ATP or [α-^{32}P]GTP is of cellular rather than viral origin.

GST22P Is Labeled with [α-^{32}P]ATP and [γ-^{32}P]ATP by CK-II. To determine whether the nucleotidylylating enzyme activity is related to CK-II, purified sea star CK-II was substituted for nuclear extract in the *in vitro* assay. The results (Fig. 2) are as follows.

(*i*) CK-II was phosphorylated in the presence of [γ-^{32}P]ATP (Fig. 2, lane 5) as previously observed (12). However, the enzyme also became phosphorylated in the presence of [α-^{32}P]ATP (lane 1). In both instances, the major amount of label was incorporated into the β subunit as previously observed.

(*ii*) GST was not labeled by CK-II in the presence of either [γ-^{32}P]ATP or [α-^{32}P]ATP.

(*iii*) GST22P and several of the truncated species were efficiently labeled by the CK-II with either [γ-^{32}P]ATP or [α-^{32}P]ATP, although it was labeled much more efficiently (≈20-fold) by [γ-^{32}P]ATP (Fig. 2, lanes 3 and 7).

(*iv*) The β subunit of CK-II was not phosphorylated in the presence of GST22P (Fig. 2, lanes 3 and 7). We were unable to determine whether the other CK-II subunits were phos-

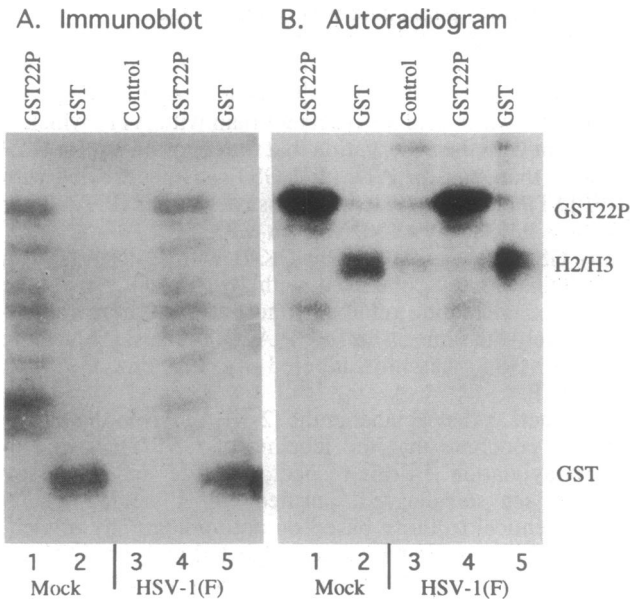

FIG. 1. Immune reactivities (*A*) and autoradiographic images (*B*) of GST and GST22P labeled by nuclear extracts and electrophoretically separated in denaturing gels. Nuclei were harvested and extracted from HeLa S3 cells harvested 20 hr postinfection or after mock infection as described (23, 24). A reaction mixture consisting of 10 μl of nuclear extract in a total of 50 μl of 50 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/7.5 μM [α-^{32}P]ATP/10 μg of GST22P was reacted for 30 min at 30°C and then electrophoretically separated on a denaturing polyacrylamide gel, electrically transferred to nitrocellulose, reacted with a polyclonal antibody to a heterologous fusion protein (GST-U_L16; J. D. Baines and B.R., unpublished studies) that recognized GST, and subjected to autoradiography as described (24). CK-II isolated from the sea star was obtained from Upstate Biotechnology (Lake Placid, NY). Substrate and source of the nuclear extract are listed above the lanes and below the image, respectively. Mobilities of the added substrates are marked on the right. Polyacrylamide gel electrophoresis, electrical transfer of electrophoretically separated polypeptides to nitrocellulose, autoradiography, and immunoblotting with polyclonal antibodies were done according to published procedures (24).

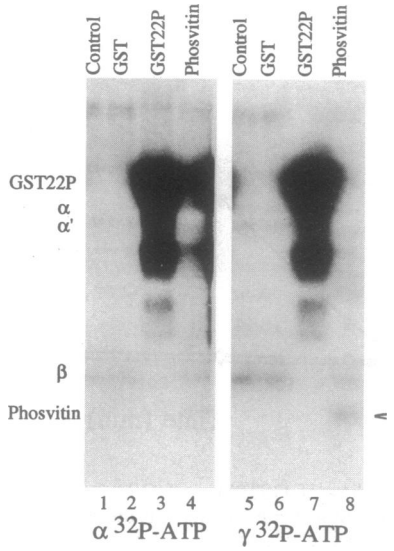

FIG. 2. Autoradiographic image of proteins labeled by CK-II with [α-^{32}P]ATP or [γ-^{32}P]ATP and electrophoretically separated in denaturing gels. Labeling reaction was identical to that described for Fig. 1 except that instead of HeLa cell nuclear extracts, sea star CK-II (Upstate Biotechnology) was added. Substrates used in these reactions are shown at the top of each lane. Positions of the GST22P and of the subunits of the CK-II are shown on the left. Position of phosvitin is marked by the arrowhead on the right. Autoradiographic images of substrates labeled with [γ-^{32}P]ATP were developed after 1 hr, whereas autoradiograms of substrates labeled by [α-^{32}P]ATP were developed after 18 hr. Both full-length and truncated GST22P were labeled and migrated as a broad band.

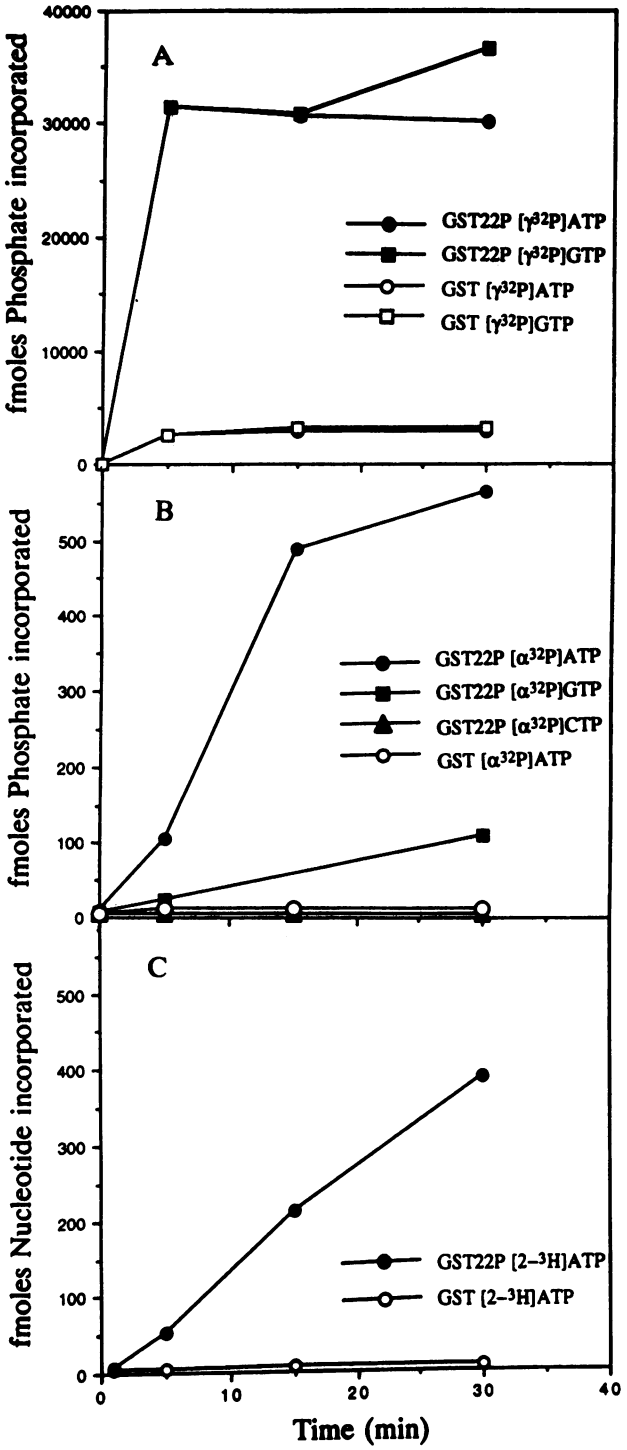

FIG. 3. Labeling of GST22P and GST by CK-II with various radioactive nucleotide triphosphates. Proteins were dot-blotted to nitrocellulose filters (Schleicher & Schuell; BA83) prewetted with phosphate-buffered saline A (140 mM NaCl/3 mM KCl/10 mM Na_2HPO_4/1.5 mM K_2HPO_4, pH 7.5) in a manifold supplied by Bio-Rad. Reaction mixtures were as described above except that they contained only 1 μg of GST22P. At stated intervals 10 μl was removed and added to 50 μl of ice-cold 0.5 M EDTA, which inhibited further labeling (24). Aliquots were stored on ice until the end of the assay, at which time they were added to the well of the dot-blotting apparatus containing a sheet of nitrocellulose. Wells were washed with phosphate-buffered saline and the nitrocellulose sheet was dried in an oven. The sheet was then cut apart, sections were dissolved in tetrahydrofuran, and then scintillation counting was done. (*A*) ^{32}P (fmol) incorporated into GST22P with [γ-^{32}P]ATP or [γ-^{32}P]GTP or into GST with [γ-^{32}P]ATP or [γ-^{32}P]GTP. (*B*) ^{32}P (fmol) incorporated into GST22P with [α-^{32}P]ATP, [α-^{32}P]GTP, or [α-^{32}P]CTP and into GST with [α-^{32}P]ATP. (*C*) ATP (fmol) incorporated into GST22P or GST by [2-^{3}H]ATP. Radioactivity retained by proteins labeled with ^{32}P was also quantified in a β-Scope (Betagen, Waltham, MA).

phorylated because they comigrated with truncated GST22P species.

(*v*) Phosvitin, a commonly used substrate of CK-II, added in equivalent amounts in place of GST22P, was labeled less efficiently than GST22P with [γ-^{32}P]ATP and to barely detectable levels with [α-^{32}P]ATP (Fig. 2, lanes 4 and 8).

We conclude that (*i*) ICP22 is a substrate for *in vitro* phosphorylation by CK-II with [γ-^{32}P]ATP. This is not unexpected since the exposed portion of the ICP22 contains at least eight consensus CK-II labeling sites. (*ii*) CK-II β subunit was not autophosphorylated in the presence of GST22P. (*iii*) ICP22 was also labeled by CK-II with [α-^{32}P]ATP, although at a lower efficiency than with [γ-^{32}P]ATP. This labeling does not arise from simple phosphorylation due to phosphate rearrangement or contamination of the [α-^{32}P]ATP with [γ-^{32}P]ATP inasmuch as phosvitin is labeled with [γ-^{32}P]ATP but not with [α-^{32}P]ATP. As demonstrated previously, at least a portion of the [α-^{32}P]ATP label represents nucleotidylylation (10).

Labeling of GST22P by CK-II with [α-^{32}P]ATP, [γ-^{32}P]ATP, or [2-^{3}H]ATP in a Filter Binding Assay. In this series of experiments, GST22P was exposed to CK-II in the presence of [α-^{32}P]ATP, [γ-^{32}P]ATP, or [2-^{3}H]ATP. To measure the ^3H label efficiently, the assays of labeled protein were done by a filter binding technique as described in the legend to Fig. 3. The results (Fig. 3) are as follows.

(*i*) The labeling of GST22P by CK-II with [γ-^{32}P]ATP or [γ-^{32}P]GTP reached maximal levels in <5 min (Fig. 3*A*). A small amount of phosphate was bound to GST.

(*ii*) Consistent with the results shown in Fig. 2, GST22P was labeled by CK-II with [α-^{32}P]ATP at a rate ≈30-fold slower than that with [γ-^{32}P]ATP (Fig. 3*B*). Labeling with [α-^{32}P]GTP was 4-fold less efficient than with ATP, which is consistent with the observation that the reported K_M for GTP is higher than that for ATP (25). GST was not labeled with [α-^{32}P]ATP or [α-^{32}P]GTP in this assay. Neither GST22P nor GST was labeled by CK-II with [α-^{32}P]CTP.

(*iii*) GST22P was labeled by CK-II with [2-^{3}H]ATP at a rate similar to that observed with [α-^{32}P]ATP. Also, the amount of nucleotide retained (fmol) after 30 min was approximately the same as for [α-^{32}P]ATP. In the same labeling interval, GST was not labeled by the enzyme with [2-^{3}H]ATP.

Inasmuch as the ^3H label in the [2-^{3}H]ATP is in the purine ring, we conclude that the labeling of GST22P is due to nucleotidylylation. In this respect, the filter binding assay with the sea star-derived, purified CK-II yielded results nearly identical to those based on autoradiography of electrophoretically separated proteins labeled with crude HeLa cell nuclear extracts. The results also show that the reaction is specific for a property of the chimeric GST22P protein that is not shared with its GST moiety.

CK-II Specifically Nucleotidylylates GST22P with [2-^{3}H]ATP but Not Substrates Known to Be Phosphorylated by the Enzyme. To determine whether nucleotidylylation was a common reaction carried out by the CK-II enzyme, labeling reactions with CK-II and [2-^{3}H]ATP were carried out with phosvitin and casein, substrates routinely used to assay phosphorylation by CK-II, and histones, a substrate phosphorylated by several cellular kinases, but not by CK-II. As shown in Fig. 4, none of these substrates was labeled in the filter binding assay.

Labeling of GST22P by [2-^{3}H]ATP Is Inhibited by Heparin. Phosphorylation by CK-II is inhibited by the addition of heparin (26) with a K_i of 20 ng/ml. As shown in Fig. 5, the

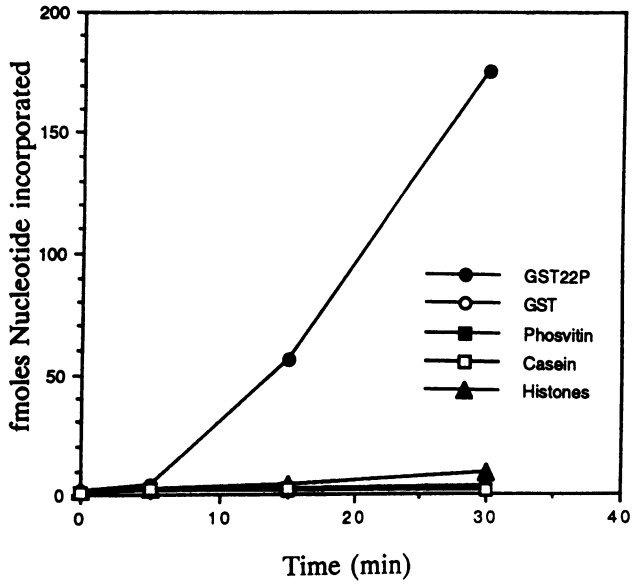

FIG. 4. Nucleotidylylation of various substrates by CK-II. Labeling reactions were identical to those described in Fig. 2. One microgram each of GST22P, GST, phosvitin, casein, and histones was reacted with CK-II in the presence of [2-^{3}H]ATP. Results are plotted as fmol of nucleotide incorporated.

nucleotidylylation of GST22P decreased dramatically at concentrations above 0.5 μg of heparin per ml. We conclude from these studies that the nucleotidylylating activity of CK-II, like that of its kinase activity, is sensitive to heparin.

DISCUSSION

In this report, we show that CK-II has an unusual activity in that it nucleotidylylates, with [α-^{32}P]ATP, [α-^{32}P]GTP, or [2-^{3}H]ATP, a protein made from the coding sequences of the α22 gene fused in-frame to the C terminus of GST. Nucleotidylylation was verified by the observation that the protein was labeled by [2-^{3}H]ATP where the ^{3}H resides in the base ring. Both the nucleotidylylating activity of CK-II and the kinase activity are sensitive to heparin. However, the two activities of CK-II appear to differ significantly with respect to substrate specificity. For example, the substrates commonly used to assay enzyme activity, phosvitin and casein, are not nucleotidylylated to a significant level, and the question whether CK-II is able to nucleotidylylate proteins other than those identified in our studies remains unanswered. Two other observations may also be significant. First, the β subunit of CK-II is not autophosphorylated in the presence of GST22P, even though GST22P is labeled to a very high level. Similarly we also noted that the host proteins designated H2 and H3, of higher apparent molecular weight than the CK-II subunits, were also not nucleotidylylated by nuclear extracts in the presence of GST22P (J.A.B., C.M., L. McCormick, and B.R., unpublished data). Second, the sequence that led to identification of the viral proteins that are nucleotidylylated—(R/P)RA(P/S)R—is different from that which signals phosphorylation by CK-II. Lastly, in lysates of cells in which infection is restricted to the expression of α proteins, only the ICP22 is nucleotidylylated *in vitro* (10). Taken together, these observations suggest that ICP22 interacts with CK-II, becomes nucleotidylylated itself, and in the process precludes CK-II from phosphorylating itself and other host proteins. Viral proteins, particularly the protein kinase specified by the U_L13 (gene) made later in infection, modify ICP22 (27, 28). Thus, CK-II or the complex between CK-II and one or more α proteins may facilitate the nucleotidylylation of additional proteins. The effect of the interaction with ICP22 on the kinase activity of CK-II has yet to be determined.

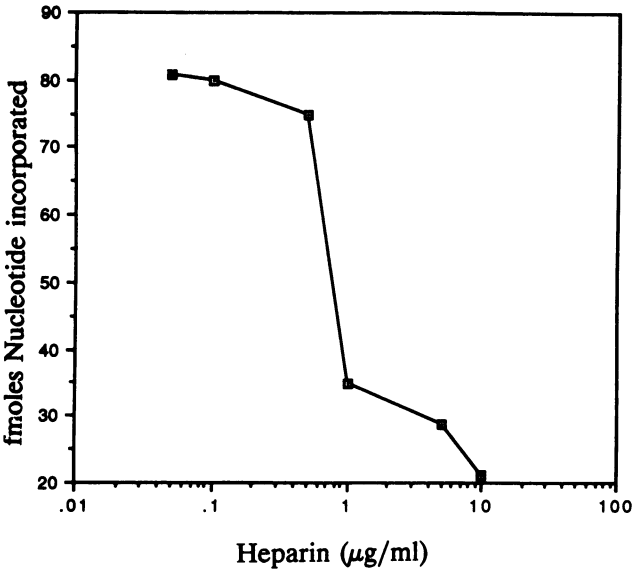

FIG. 5. Effect of heparin on labeling of GST22P by CK-II. Labeling reactions were identical to those described in Fig. 2 except that the reactions were terminated at 15 min by the addition of 20 μl of ice-cold 0.5 M EDTA. The entire 70 μl was added to the dot-blot apparatus and processed as described in the legend to Fig. 3. Activity was graphed as fmol of nucleotide incorporated vs. amount of heparin added to the assay mixture (μg/ml) plotted on a logarithmic scale.

ICP22 is required for the expression of a subset of viral genes (28, 29). ICP22 is also phosphorylated by two protein kinases specified by the U_L13 and U_S3 genes (27). Whereas the kinase specified by the U_S3 gene is not required for the function of the ICP22, the phenotype of U_L13 null mutants cannot be differentiated from that of $\alpha22^-$ mutants in cultured cells (28).

CK-II is a multifunctional eukaryotic enzyme found in the cytosol, nucleus, mitochondria, and membranes (25, 30–32). Among the most interesting substrates are nuclear proteins involved in cell replication and oncogenesis such as *fos* (33), *myc* (34), and p53 tumor suppressor protein (35). Also, CK-II has been reported to phosphorylate viral proteins such as papillomavirus E7 protein (36, 37), adenovirus E1A protein (33), simian virus 40 large T antigen (38), and a cellular protein that has sequence similarity, including a consensus CK-II phosphorylation site, to ICP4, the major HSV-1 regulatory protein (39). It has been reported that phosphorylation of eukaryotic RNA polymerase II by CK-II is required for transcription (40, 41). It is noteworthy that ICP4 protein is nucleotidylylated (24), presumably by CK-II, and that the major subunit of the RNA polymerase II is modified with the involvement of one of the α proteins in HSV-1-infected cells (42). Studies to determine the role of CK-II and the nucleotidylylation of α proteins on the function of the RNA polymerase in HSV-1-infected cells remain to be done.

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