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STUDIES ON THE MECHANISM OF ACTIVATION OF MITOTIC HISTONE H1 KINASE

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SUMMARY: A chromatin-associated histone H1 kinase has been detected in synchronized Novikoff hepatoma cells. Enzyme specific activity increased 4 to 6-fold from late G-2 to mid-metaphase, then decayed exponentially ($T_{1/2}$, 28.5 min) to the interphase level. Extracts of the mitotic kinase retained the ability to decay in vitro at 37°C but not at 0°C ($T_{1/2}$, 24 min), under conditions in which interphase activity was stable. Sedimentation rates in sucrose density gradients of interphase and mitotic enzymes (before and after decay) were identical. Purification did not alter the rate of enzyme decay. However, high ionic strength prevented decay of crude but not purified preparations of mitotic enzyme. The results are discussed in terms of an allosteric mechanism for reversible activation of enzyme activity.

INTRODUCTION: A chromatin-bound protein kinase with high specificity for histone H1 was originally discovered by Lake and Salzman in V 79 Chinese hamster cells (1). This enzyme is broadly distributed in growing cells (1-3) but is absent from proliferatively-static tissues (4) and has thus been termed growth-associated histone kinase (HKGR). The partially purified enzymes from mouse Ehrlich ascites cells (5) and from Chinese hamster cells (6) have been shown to preferentially phosphorylate histone H1 at the same sites found to be phosphorylated in vivo. The level of HKGR activity increases several-fold in synchronized mammalian (1) or Physarum (2) cells close to the onset of mitosis and this is accompanied by a large increase in the phosphorylation of H1 in vivo (2,7,8). A number of studies strongly suggest that mitotic H1 phosphorylation may initiate (2,9) or at least participate in (8) the normal process of chromosome condensation. The mechanism by which HKGR activity increases as cells enter mitosis is of interest in understanding the regulation of cell division. It has recently been reported that the mitotic increase in Physarum HKGR represents activation of pre-existing enzyme rather than de novo synthesis (10). Based on these results, we have begun an investigation of possible mechanisms of activation and inactivation of the enzyme in mammalian cells. We report here the characterization of the cell cycle dependency of HKGR in synchronized Novikoff hepatoma cells and show that cell-free preparations of the mitotic enzyme exhibit the ability to return to the interphase level of activity in a manner which appears to be similar to that observed in intact cells. The characteristics of this phenomenon suggest a specific model for enzyme activation in vivo.

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METHODS: Novikoff hepatoma cells were grown and synchronized as described previously (11). HKGR activity was measured at various times during the cell cycle in homogenates of 1 to 2 X 10^7 cells previously frozen as pellets (11) at -70°C. The use of frozen versus fresh cells was without effect on observed HKGR activity at any time during the cell cycle. Cell pellets were homogenized with a Brinkman Polytron (15 sec at half-maximum setting) in 2 ml of ice-cold 75 mM NaCl and 24 mM EDTA (pH 8.0). All operations were conducted at 0 to 4°C. The insoluble HKGR-containing chromatin complex was isolated by repeated centrifugation at 1500 X g for 5 min in 2 changes of 50 mM Tris.HCl, pH 8.0. The final washed chromatin pellet was resuspended in 1.5 ml of Tris. HCl, pH 8.0 and assayed for kinase activity using calf thymus H1 (12) as substrate. Assays were conducted in a total volume of 0.1 ml containing 50 mM Tris. HCl, pH 8.0, 1 mM DTT, 5 mM $MgCl_2$, 1.0 mg/ml histone, 0.5 mM [^{32}P-γ] ATP (2 to 5 X 10^4 cpm/nmole) and up to 0.05 ml of chromatin suspension containing 5 to 25 mg protein. Reactions were initiated by the addition of enzyme and were conducted at 37°C for 20 min. Assays were terminated by a modification (13) of the filter paper method of Corbin and Reimann (14). Phosphorylated product was quantitated in a Nuclear Chicago thin window planchet counter. Reactions were corrected for endogenous chromatin phosphorylation in the absence of added substrate and were linear with respect to time and enzyme concentration. Analysis of specific phosphorylation sites in H1 (5) indicated that HKGR was the major if not the only H1 kinase activity present in washed chromatin from Novikoff hepatoma cells under these conditions.[1]

To study decay of mitotic HKGR in vitro, the insoluble washed chromatin pellet or the solubilized enzyme (see below) was resuspended or diluted into a stabilizing buffer composed of 10 mM Tris. HCl (pH 7.5), 140 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and 0.5 mg/ml bovine serum albumin and incubated under various conditions. In experiments where salt concentration was varied, NaCl or KPO_4 buffer (pH 7.5) was substituted for KCl as indicated in individual experiments. Ten ul aliquots of incubated enzyme were assayed as described above for 10 min. The effect of incubation on enzyme activity was studied at three levels of purification, namely, the insoluble enzyme-chromatin complex (ca. 10-fold purification referenced to the original crude homogenate), 106,000 X g soluble supernatant from the 0.4 M salt (NaCl) extract of chromatin (ca. 30 to 40-fold purification), and the 0.2 M (KPO_4) calcium phosphate gel eluate (ca. 100 to 150-fold purification). The general procedure used to purify HKGR was essentially as described previously (4). Protein was measured by the method of Lowry et al. (15) using bovine serum albumin as the standard. One unit of enzyme activity is defined as 1 µmole ^{32}P transferred to substrate/h. Unless otherwise indicated, all data are averages of independent duplicates and are representative of several experiments in each case.

RESULTS: The cell cycle dependency of HKGR in Novikoff hepatoma cells is shown in Fig. 1. With the exception of the mitotic period, the specific activity of HKGR was essentially constant throughout the cell cycle, thus conforming to a linear pattern of enzyme synthesis (16). However, a 4- to 6-fold increase in kinase activity was observed as cells proceeded from late G-2 to colcemid-blocked mid-metaphase (Fig. 1A and 1B). The rise in enzyme activity was proportional to and dependent on the mitotic index at any given time in the presence or absence of colcemid, thus indicating that colcemid itself did not produce an artifactual activation of the enzyme in vivo. In additional experiments, the rise in HKGR activity was found to precede the rise in metaphase-arrested cells by 24.7 ± 5.4 min ($P < 0.01$; $n = 6$). Since the duration of prophase in these cells has been estimated to be approximately 13 min (Zeilig, C.E., unpublished observations), it is clear that the cell cycle-dependent increase in HKGR represents a late G-2 rather than an early mitotic event, in agreement with the findings for the Physarum enzyme (2). Reversal of

[1] Zeilig, C. E. and Langan, T. A., unpublished results.

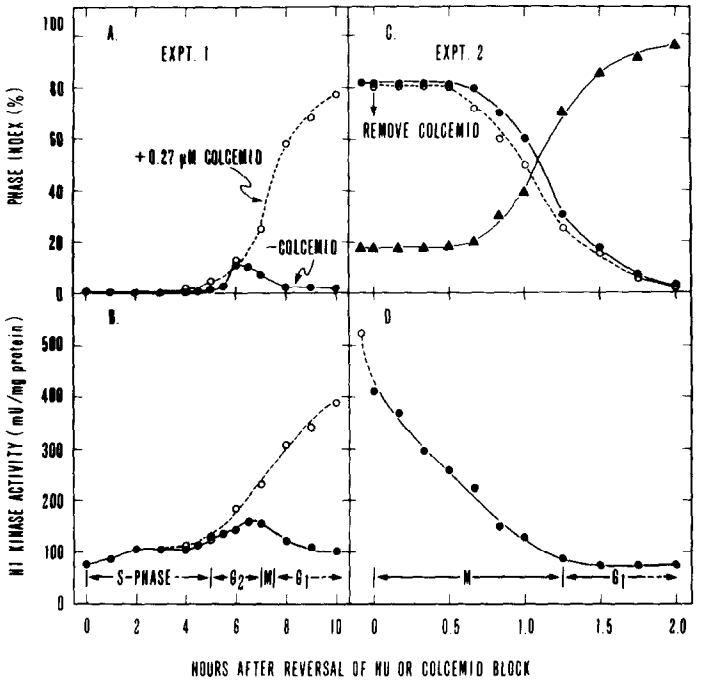


Figure 1. Chromatin-associated histone H1 kinase levels in synchronized Novikoff hepatoma cells. Synchronization and monitoring of cell cycle traverse was as described (11). See Methods for conditions of enzyme assay. Panels A and B: mitotic index (A) or kinase activity (B) as synchronized cells enter mitosis following reversal of a double hydroxyurea block in the presence (---o---) or absence (—●—) of 0.27 μM colcemid. Panels C and D: mitotic index (C) or kinase activity (D) of cells exiting mitosis. Cells were synchronized by a single hydroxyurea block followed by reversible colcemid arrest. Panel C:—●—, % mitotic cells; ---o---, % metaphase cells; —▲—, % interphase cells. Panel D: kinase activity before (---o---) or after (—●—) reversal of colcemid block.

the colcemid block (Fig. 1C and 1D) led to a rapid, near exponential decay of mitotic HKGR (mHKGR) down to the interphase level (iHKGR) 40 min prior to the completion of metaphase with a calculated $T_{½}$ of 28.5 min (interphase activity subtracted).

In order to determine whether a similar decay phenomenon could be reproduced in cell-free conditions, washed chromatin pellets were prepared from colcemid-arrested mitotic cells, incubated for varying period of time, and assayed for HKGR activity (Fig. 2). Incubation of mHKGR at 37°C for 2 h led to a fairly rapid inactivation of activity. Such inactivation did not occur at 0°C. In contrast, iHKGR activity was stable under identical conditions at 37°C (Fig. 2) and this stability was unaffected by admixture with mHKGR (data not shown). Inclusion of Triton X-100 (0.1%) during the incubation period was found to stimulate the rate and extent of mHKGR inactivation but had little effect on iHKGR stability. These results indicate a marked, temperature-sensitive, difference in the stability of mHKGR versus iHKGR.

Preparations of mHKGR which had been solubilized by extraction in 0.4 M NaCl and then separated from the bulk of chromatin protein by centrifugation were also found to

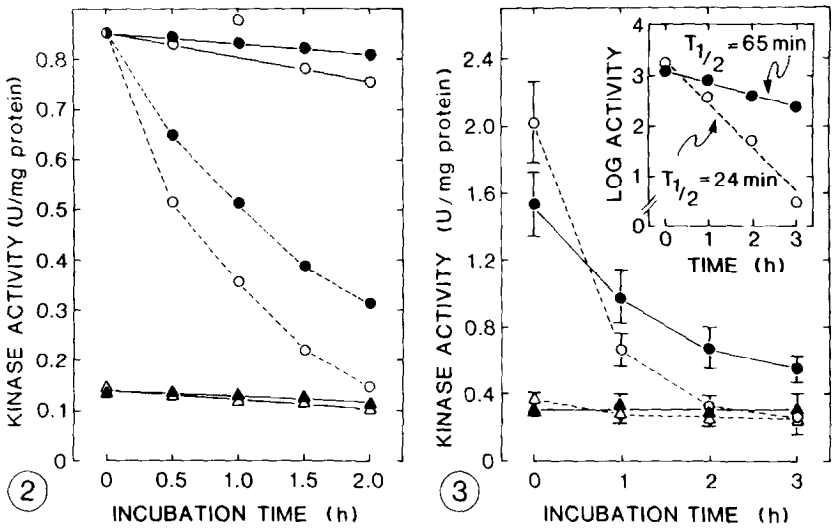


Figure 2. Comparison of chromatin-bound interphase and mitotic histone H1 kinase stability in vitro. Mitotic enzyme was from cells synchronized as described in the legend to Fig. 1C. Interphase enzyme was from randomly growing cells exposed to hydroxyurea for 9h and was from the same population used to obtain mitotic cells. See Methods for conditions of enzyme incubation and assay. Incubation buffer contained 140 mM KCl. Circles, mitotic enzyme incubated at 0°C (————) or at 37°C (--------) in the presence (○) or absence (●) of 0.1 % Triton X-100. Squares, interphase enzyme with (□) or without (■) 0.1 % Triton X-100.

Figure 3. Comparison of stability of solubilized mitotic and interphase histone H1 kinase activities. Incubation buffer contained 200 mM NaCl. Circles, mitotic activity with (---○---)and without (—●—) 0.1 % Triton X-100. Triangles, interphase activity with (---△---) and without (—▲—) 0.1 % Triton X-100. Inset, logarithmic plot of mitotic enzyme activity as a function of time at 37°C (interphase activity subtracted) in the presence (---○---) or absence (—●—) of 0.1% Triton X-100. Data represent the mean ± SEM of 6 experiments.

decay in a manner similar to that observed with chromatin-bound mHKGR (Fig. 3). Decay of solubilized mHKGR was exponential (Fig. 3, inset) and the rate of decay was stimulated 2.7-fold by Triton X-100 resulting in a $T_{½}$ (24 min) which was very similar to that observed in vivo. In the presence of Triton X-100, decay of mHKGR proceeded to a level essentially equal to that exhibited by iHKGR.

In the course of these experiments high concentrations of NaCl (or KCl or KPO_4 buffer of equivalent ionic strength) were found to prevent decay of mHKGR and to activate iHKGR to a limited extent. The effects of salt concentration on enzyme activity were therefore examined more systematically (Fig. 4). Chromatin extracts of iHKGR and mHKGR were diluted and incubated for 60 min at 37°C in salt concentrations ranging from 0.05 to 1 M. A progressive inhibition of mHKGR decay was observed which became complete at 0.8 M NaCl (Fig. 4A). At this salt concentration, mHKGR decay could be arrested completely for at least 3 h (see also Fig. 7). It is notable that the ability of 0.8 M salt to prevent decay of mHKGR was completely overcome by the addition of 0.1% Triton X-100 (data not shown) and rates of decay comparable to those observed previously

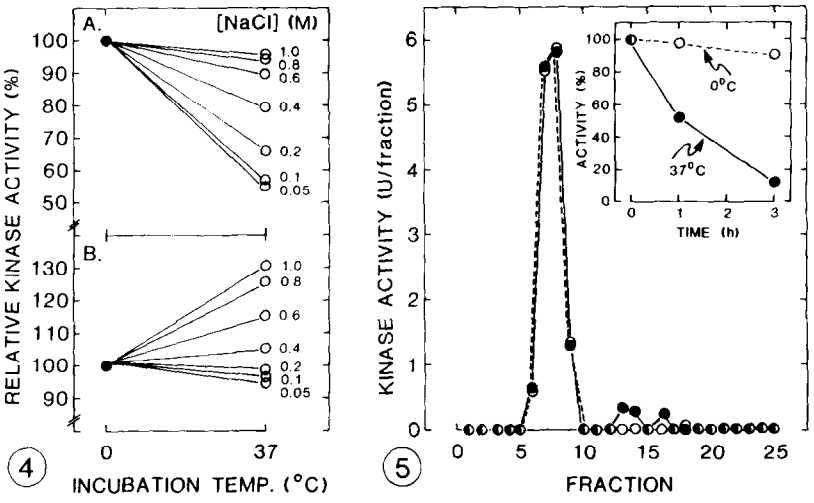


Figure 4. Effect of ionic strength on interphase and mitotic histone H1 kinase activity. Solubilized mitotic (Panel A) or interphase (Panel B) kinases were diluted into incubation buffer containing NaCl concentrations as indicated and incubated for 60 min at 0°C (●) or 37°C (○). Kinase activity at 37°C is expressed relative to that observed at 0°C for each NaCl concentration to compensate for non-specific inhibitory effects of NaCl on catalytic activity (ca. 15% at 1M NaCl) which were independent of incubation time.

Figure 5. Sucrose density-gradient centrifugation of mitotic histone H1 kinase. Solubilized (see Methods) mitotic kinase which had been incubated under conditions shown in Fig. 3 at 0°C (---o---) or 37°C (—●—) for 3 h (see Inset) was loaded onto a 12.5 ml 5-20% linear sucrose gradient containing standard incubation buffer with 200 mM NaCl and subjected to isokinetic centrifugation at 20,000 x g for 20 h at 0°C. Twenty µl of each 0.5 ml fraction was then assayed for kinase activity. Top of the gradient is to the left. ---o---, 0°C incubated enzyme; —●—, 37°C incubated enzyme (units have been multiplied by 2 for graphical comparison).

in the presence of lower salt concentrations and Triton X-100 were observed (see Fig. 3). In contrast, with iHKGR, a modest concentration and temperature-dependent activation was observed at all concentrations of NaCl tested (Fig. 4B). When tested under the same conditions, the chaotropic salt, NaSCN, did not prevent decay of mHKGR. However, NaSCN directly inhibited catalytic activity of mHKGR and iHKGR, with identical K_i's of 0.35 M for both enzymes (data not shown).

The possibility that the decrease in mHKGR activity at 37°C might involve a large change in molecular weight was assessed in the following experiment (Fig. 5). Chromatin extracts of mHKGR, before and after decay had been allowed to occur (see inset, Fig. 5), were subjected to isokinetic linear sucrose density gradient centrifugation. No significant change in sedimentation rates was observed in the enzyme preparation which had been allowed to decay. In addition, the sedimentation rate of mHKGR did not differ from that exhibited by iHKGR (data not shown).

In order to determine the characteristics of more highly purified enzyme, chromatin extracts of iHKGR and mHKGR were purified by ammonium sulfate and calcium phosphate gel fractionation (see Methods and ref. 4). Recovery and fold purification at

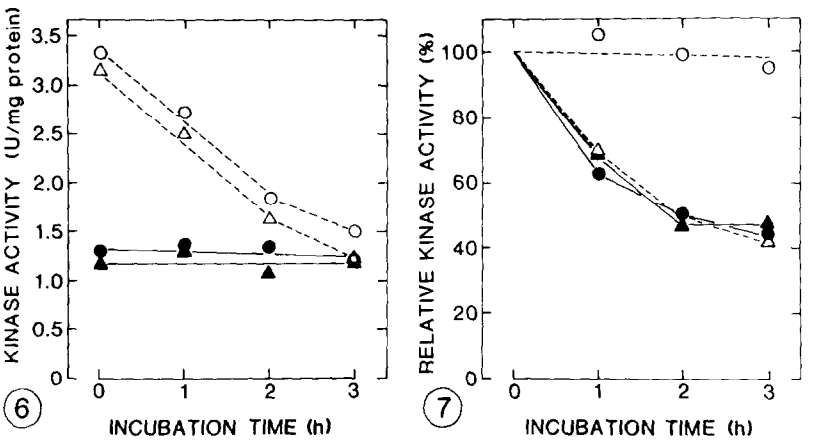


Figure 6. Comparison of the stability of mitotic and interphase histone H1 kinase in the presence of high or low ionic strength. Solubilized kinases were subjeced to ammonium sulfate and calcium phosphate gel fractionation (see Methods). Enzyme was eluted from the gel in 0.2M KPO_4, buffer, pH 7.5, and diluted into low (50 mM KPO_4, 100 mM NaCl; μ=233.5 meq) or high (50 mM KPO_4, 666.5 mM NaCl; μ= 800 meq) ionic strength incubation buffer and incubated for various times at 37°C prior to assay. ---o---, —●—, mitotic and interphase kinases, respectively, in low ionic strength buffer; ---Δ---, —▲—, mitotic and interphase kinases, respectively in high ionic strength buffer.

Figure 7. Effect of purification on stability of mitotic H1 kinase in the presence of high ionic strength. Solubilized mitotic kinase extracted from washed chromatin (o,●) or purified further through the calcium phosphae gel elution step (Δ,▲) (see Methods) was diluted into low (————) or high (--------) ionic strength buffer as described in the legend to Fig. 6 and incubated for various times at 37°C prior to assay. Specific activity at 0-time has been equalized to that observed in each preparation maintained at 0°C prior to assay.

each stage of purification was found to be the same for both iHKGR and mHKGR. At this level of purity, the gel eluate of mHKGR retained the capacity to decay at rates at least as great as those observed in less purified preparations (Fig. 6). However, the ability of high ionic strength to prevent decay of mHKGR in chromatin extracts was completely lost in these more highly purified preparations.

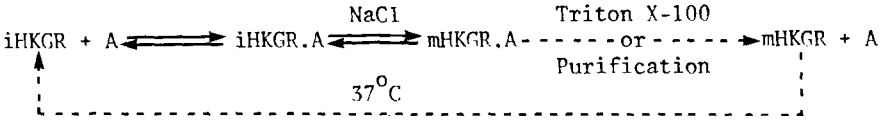
A direct comparison of the rate of decay of mHKGR in chromatin extracts with the more highly purified gel eluate preparations at high and low ionic strength is shown in Fig. 7. These results clearly demonstrate that there is no significant effect of purification per se on the rate of mHKGR decay in low ionic strength buffer. As observed previously, purification of mHKGR resulted in an enzyme preparation in which decay was no longer prevented by high ionic strength.

In additional experiments, HKGR activity or decay has been found to be independent of cyclic nucleotides (cAMP, cGMP, cCMP) EGTA, Ca^{2+}, or purified calmodulin, cGMP-dependent protein kinase, frog oocyte maturation promoting factor, and various phosphatase inhibitors (NaF, Na_2HAsO_4, Na_2MoO_4). Attempts to activate iHKGR by mixing with cytosol extracts from mitotic cells have also proven unsuccessful to date.

DISCUSSION: Interphase and mitotic HKGR share a number of identical physical and catalytic properties. Analysis of tryptic phosphopeptides from histone H1 phosphorylated by iHKGR and mHKGR has shown that both enzymes exhibit identical site-specificities (6)[1]. As shown previously by Lake (6), both enzymes also display identical substrate specificities. These observations argue persuasively that both forms of HKGR share a common catalytic domain. The physical characteristics of both enzymes must also be nearly identical since both enzymes are found to: [1] co-purify through a common fractionation scheme;[2] co-migrate in sucrose gradients (Fig. 4) and gel filtration (6); and, [3] be inhibited by identical concentrations of NaSCN. However, the two enzymes differ markedly in their stabilities at 37°C. This finding clearly excludes the possibility that the increase in HKGR at mitosis results simply from de novo synthesis of the same enzyme present in interphase cells. On the other hand, because of the number of fundamental similarities between the two enzymes, it seems unlikely that the elevated mHKGR activity results from the de novo synthesis of an entirely different enzyme. These observations lend strong support for the conclusion that iHKGR and mHKGR represent interconvertible forms of the same enzyme. Based on measurements of the rate of enzyme synthesis using a deuterium-density labeling technique, Michelson et al. (10) have also concluded that elevation of enzyme activity at mitosis in Physarum does not involve synthesis of new enzyme.

The selective, temperature-dependent decay of mHKGR to a level identical with the interphase activity (see Fig. 2, 3, and 6) could be explained by one of three possible mechanisms: [1] enzymatic reversal of a covalent modification;[2] dissociation of a small molecular weight, non-covalent activator; or, [3] non-specific thermal or proteolytic denaturation of mHKGR but not iHKGR. It is unlikely that decay of mHKGR occurs as a result of proteolysis since the rate of decay was not altered at increasing levels of purification. Moreover, the inability of mitotic chromatin to induce lability of iHKGR when the two enzymes are incubated together, the fact that mHKGR decays only to the interphase level and not lower, and the selective ability of high ionic strength to prevent decay of mHKGR in chromatin extracts but not in purified preparations, are all incompatible with non-specific proteolysis of mHKGR. Non-specific thermal denaturation of mHKGR also seems unlikely since iHKGR is stable under the same incubation conditions (Figs. 2, 3, and 6). The finding that the rate of mHKGR decay is unaffected at successively higher levels of purity also argues against a role for a specific, extrinsic, enzymatic inactivating factor in mediating the temperature-dependent decline in mHKGR activity. Finally, the fact that the ability of high ionic strength to stabilize mHKGR at 37°C is lost upon purification suggests the loss of some factor from the mitotic enzyme. For these reasons, we believe the second possibility, namely, dissociation of a small molecular weight activator, is the most likely explanation for the decay of mitotic enzyme activity observed in the present experiments. According to this mechanism,

enzyme activation would occur upon binding of an activator, A, to iHKGR followed by a subsequent conformational shift to the active form of the enzyme as diagrammed below:



On the basis of this model, enzyme activity would normally be controlled in vivo by the synthesis and degradation of A. The in vitro effects of temperature and salt on inactivation of the mitotic enzyme suggest that the conformational shift is temperature dependent, and that the mHKGR.A complex is stabilized by high salt. Purification or Triton X-100 treatment might lead to the loss of A and the formation of a labile form of the enzyme, free mHKGR, which is not stabilized by high salt (see Figs. 3 and 7). There are a number of predictive features of this model which are amenable to experimental verification. Further studies to test this hypothesis and to ascertain conditions for detecting an activator of HKGR in mitotic cells are being pursued.

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