ADDITIONAL EVIDENCE THAT THE HEMIN-CONTROLLED TRANSLATIONAL REPRESSOR FROM RABBIT RETICULOCYTES IS A PROTEIN KINASE

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SUMMARY. Recent reports have suggested that the hemin-controlled translational repressor (HCR) which mediates the hemin control of protein synthesis in reticulocyte lysates, acts as a specific protein kinase, phosphorylating a subunit of the Met-tRNA$_f$ binding factor (IF-1). We have found that crude and highly purified HCR can phosphorylate a 38,000 molecular weight component of IF-1, but that crude prorepressor (the precursor of HCR), which is not inhibitory, does not phosphorylate this component. Prolonged warming of the prorepressor induces the formation of the inhibitor and the protein kinase that phosphorylates the 38,000 molecular weight protein, and the formation of both is blocked by hemin. In addition, a brief incubation of the prorepressor with N-ethylmaleimide, which produces maximal inhibitory activity within 5 minutes, also induces formation of the protein kinase. These findings suggest that HCR and the protein kinase are the same protein and provide additional support for the concept that HCR controls protein synthesis by phosphorylating the Met-tRNA$_f$ binding factor.

Considerable evidence supports the concept that the regulation of protein synthesis by hemin in rabbit reticulocyte lysate is mediated by the formation of an inhibitor of polypeptide chain initiation, termed the hemin-controlled translational repressor (HCR) (1-5). This translational inhibitor is a high molecular weight protein (2,6) that is formed in the absence of hemin in three distinct stages (7) from a precursor (prorepressor) in the post-ribosomal supernatant fraction. HCR appears to act either by blocking formation of a complex between Met-tRNA$_f$ and the 40 s subunit (4,5,8) or by blocking the formation of 80 s initiation complexes from 40 s complexes (9,10). The inhibitory effect of hemin deprivation or HCR can be partially overcome by the initiation factor that promotes binding of the initiator tRNA to the 40 s subunit (here termed IF-1) (2,11-13), as well as another initiation factor (IF-2) that promotes formation of 80 s initation complexes from 40 s complexes (M. Gross, manuscript submitted). In addition, the inhibition of protein synthesis due to hemin deprivation or HCR can be completely reversed by a supernatant factor from reticulocyte lysate, that is distinct from IF-1 and IF-2 (14,15).

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Abbreviations used: HCR (hemin-controlled translational repressor); HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid); NEM (N-ethylmaleimide); SDS (sodium dodecyl sulfate).

Recent reports have suggested that HCR may act as a specific protein kinase, phosphorylating a 38,000 molecular weight (38K) subunit of IF-1 (16,17, T. Hunt, personal communication). We present here additional data that strongly support this hypothesis. The possible effect of phosphorylation on the activity of IF-1 is discussed.

METHODS AND MATERIALS

Cell Free Protein Synthesis: Reticulocyte lysate was prepared from phenylhydrazine treated rabbits as described (1). Samples prepared for protein synthesis contained 5 volumes of lysate, 3 volumes of master mix and 3 volumes of test solution or water. The master mix contained the following components in amounts to yield the indicated concentrations in the final reaction mixture: KCl (75 mM); $MgCl_2$ (2 mM); ATP (0.5 mM); GTP (0.2 mM); creatine phosphate (15 mM); creatine phosphokinase (45 units/ml); L-[1-^{14}C]leucine (0.3 mM, 5 Ci/mole); and a mixture of the 19 other amino acids at concentrations corresponding to the amino acid composition of rabbit globin. Hemin was added at a final concentration of 20 μM. The incorporation of leucine into protein was determined as described (7). The inhibitory activity in preparations of HCR and prorepressor was measured by adding dilutions of the inhibitor to 110 μl, protein synthesizing samples incubated in the presence of hemin for 90 minutes at 34°. One unit of HCR inhibitory activity represents the amount required to reduce, by 50%, the difference in protein synthesis between no HCR and an amount that produces maximal inhibition (1).

Preparation of Prorepressor and HCR. All operations were at 0 - 5°. The post-ribosomal supernatant fraction was obtained by centrifuging lysate at 250,000 x g avg for 4.5 hours in the 60 Ti rotor. Prorepressor was prepared by chromatographing 12 ml of the supernate on a 2.5 x 48 cm CM-Sephadex column, equilibrated in 6 mM HEPES, pH 7.2 (Buffer A). The column was washed with Buffer A, and the fraction not bound to the column (freed of hemoglobin) was pooled, concentrated to one-third the volume of the original supernate, and stored in small aliquots in liquid nitrogen. Crude HCR was prepared by warming prorepressor in the absence of hemin for 16 hours at 34°C (6). It had a potency of 440 units/mg and was stored in small aliquots at -90°. Prorepressor had only 2% of the inhibitory activity of crude HCR. Highly purified HCR was prepared as previously described (18) and had a potency of 27,000 units/mg. HCR was also rapidly generated by incubating 250 μl of prorepressor with 5 mM N-ethylmaleimide (NEM) for 5 minutes at 34°, followed by 5 minutes of further incubation with 5 mM dithiothreitol (6). The sample was cooled to 0° and filtered through a 20 x 0.7 cm Sephadex column, equilibriated in Buffer A containing 28 mM KCl. The fraction excluded from the column was pooled (final volume equal to the original post-ribosomal supernate) and stored in small aliquots at -90°. It had a potency of 420 units/mg.

Preparation of IF-1: IF-1 (the Met-tRNA$_f$ binding factor) was isolated from the 0.55 M KCl wash of reticulocyte ribosomes by chromatography on DEAE cellulose, refractionation on DEAE cellulose, and chromatography on hydroxyapatite as described (M. Gross, manuscript submitted). It had a potency of 120 units/mg where one unit represents the amount of factor required to bind one picomole of [^{35}S]Met-tRNA$_f$ to a millipore filter in the presence of GTP.

Determination of Protein Kinase Activity: Protein kinase activity was measured in 100 μl samples containing 6 mM HEPES, 6 mM $MgCl_2$, 0.4 mg/ml of IF-1, the indicated amounts of HCR, and 0.1 mM ATP-γ-[^{32}P] (200 mCi/mMole). 3':5'-cyclic AMP was not included in the protein kinase assay, because it did not enhance the phosphorylation of the 38K subunit of IF-1 by HCR preparations, in agreement with Levin et al. (16) and Kramer et al. (17). Samples were incubated at 34°, usually for 10 minutes, and the reactions were stopped by the addition of 20 μl of denaturing solution (50 mM Tris-HCl, pH 8.0, 5% sodium

dodecylsulfate (SDS), 50% sucrose, 5 mM EDTA, 160 mM dithiothreitol, and 0.1 mg/ml pyronin Y). After incubation at 57° for 30 minutes, each sample was applied to a 0.6 x 8.5 cm, 5% polyacrylamide gel containing 0.2% SDS, and electrophoresed at about 3.5 mA/gel for 3.5 hours. Gels were fixed and stained overnight in 0.02% (w/v) coomassie blue in 10% acetic acid -25% isopropanol and then destained over the next 24 hours, first with 10% acetic acid -10% isopropanol and then with 10% acetic acid. After each gel was scanned at 550 nm, 1 mm slices were prepared and counted at 30% efficiency with 2 ml of water in a Packard model 3380 liquid scintillation spectrometer using the cerenkov radiation (19). This simplified technique was justified because the activity measured by cerenkov radiation was directly proportional to the input [^{32}P] radioactivity, and the radioactivity obtained when selected gel slices were dissolved with H_2O_2 and then counted with scintillation fluid was proportional to the radioactivity determined by counting the cerenkov radiation.

Master mix reagents and hemin were from Sigma. L-[1-^{14}C]leucine and [^{32}P]-γ-ATP were purchased from New England Nuclear. HEPES, NEM and dithiothreitol were from Calbiochem. Molecular weight standards and CM-Sephadex were from Pharmacia.

RESULTS

We felt that a crucial test of the hypothesis that HCR blocks polypeptide chain initiation by phosphorylating a subunit of the Met-tRNA$_f$ binding factor (IF-1) would be to determine whether HCR and the protein kinase are formed in parallel. The preparation of IF-1 used in these experiments, although not homogeneous, did contain two prominent bands on SDS-polyacrylamide gels, corresponding to apparent molecular weights of 52,000 and 38,000 (Figure 1, Frame A) that appear to represent the subunits of the Met-tRNA$_f$ binding factor as reported by Safer et al. (20). When IF-1 was incubated with γ-[^{32}P]-ATP in the absence of HCR (Figure 1, Frame A), the 52,000 molecular weight protein became phosphorylated, apparently due to the action of a protein kinase in the IF-1 preparation, but no peak of radioactivity was associated with the 38,000 molecular weight (38K) component. Incubation of crude HCR with γ-[^{32}P]-ATP in the absence of IF-1 (Frame B) showed no significant labeling in the 38K region. Incubation of IF-1 with crude HCR (Frames C and D), however, showed extensive, dose-dependent labeling of the 38K band. In contrast, incubation of IF-1 with prorepressor (Frames E and F) was associated with only a low level of phosphorylation of the 38 K subunit. This demonstrates that the conversion of prorepressor to HCR by warming in the absence of hemin is associated with the formation, in parallel, of the protein kinase that phosphorylates the 38 K component of IF-1. When HCR was generated by a 5 minute incubation of prorepressor with NEM (Frame G), the formation of inhibitory and protein kinase activities was again induced in parallel. In addition, HCR and the protein kinase were found to copurify (Frame H), in agreement with recent reports by Levin et al.(16) and Kramer et al.(17). These findings add strong support to

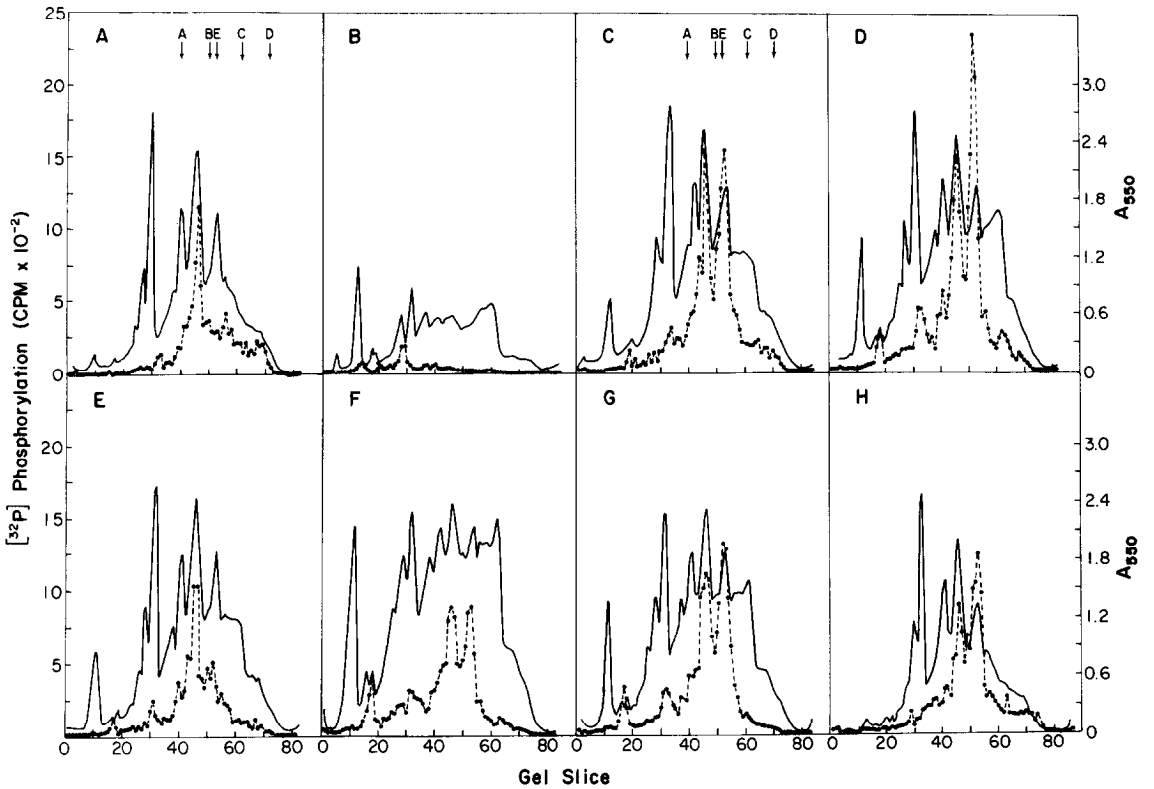

Figure 1. A comparison of the protein kinase activity of prorepressor and crude, highly purified, and NEM prepared HCR.

Standard protein kinase assay samples (100 µl) contained the following preparations of HCR: (A) no added HCR; (B) 10 units of crude HCR (23 µg), but no IF-1; (C) 5 units of crude HCR (11.5 µg); (D) 10 units of crude HCR (23 µg); (E) 11.5 µg of prorepressor; (F) 58 µg of prorepressor; (G) 10 units of NEM prepared HCR (24 µg); and (H) 10 units of highly purified HCR (0.4 µg). Samples were incubated at 34° for 10 minutes, electrophoresed on SDS-polyacrylamide gels, and stained, scanned, and counted as described in the methods. Radioactivity is depicted by the dashed line, and absorbance, by the smooth tracing. Moleceular weight markers, run in parallel, migrated at the position indicated by the arrows: A (bovine serum albumen); B (ovalbumen); C (chymotrypsinogen); and D (apomyoglobin). Arrow E indicates the 38,000 molecular weight position determined from a plot of the four protein standards.

the concept that HCR and the protein kinase are the same protein. One additional finding shown in Figure 1, that NEM prepared HCR and highly purified HCR have only about one-half as much protein kinase activity as crude HCR, formed by prolonged warming, when all preparations are normalized to the same inhibitory activity, is considered in detail later.

Another important test of the protein kinase model of HCR action was to

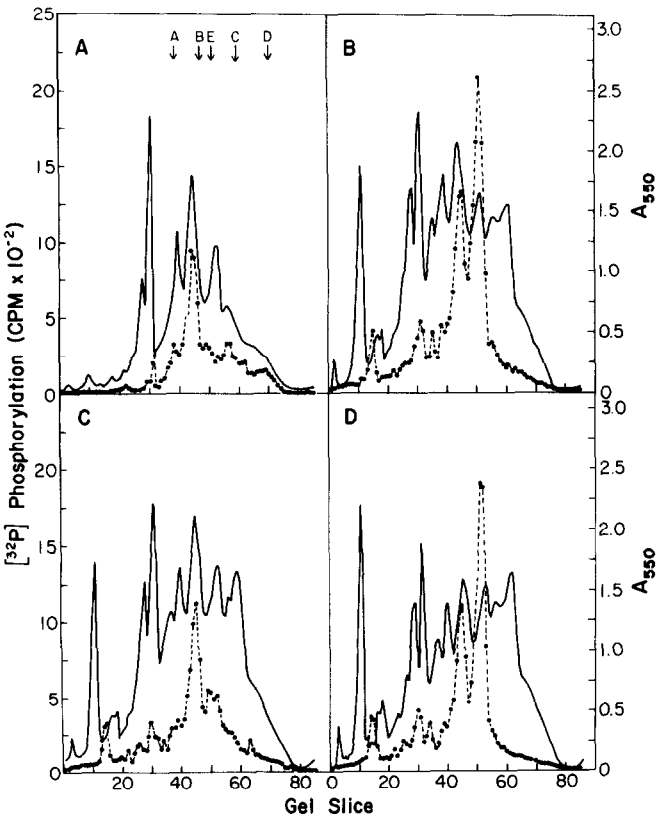

Figure 2. Effect of hemin on the formation and activity of the protein kinase produced when the prorepressor is converted to HCR.

Standard protein kinase assay samples (100 µl) contained no added HCR (A) or 35 µg of prorepressor, warmed at 34° for 6 hours in the absence of hemin (B, containing 5 inhibitory units), in the presence of 0.4 nMoles of hemin (C, 0.7 inhibitory units), or warmed in the absence of hemin and then given 0.4 nMoles of hemin (D, 5 inhibitory units). Hemin was at a final concentration of 40 µM in the preincubation of prorepressor and 4 µM in the protein kinase assay. We have previously shown that the stimulation of protein synthesis in the lysate and the suppression of HCR formation by hemin is dependent upon the ratio of hemin to protein and not the absolute concentration of hemin (6). The remainder of the procedure was as described in the legend to Figure 1.

determine whether hemin would prevent the formation of protein kinase activity as it does inhibitory activity, when the prorepressor is warmed. The results in Figure 2 demonstrate that the formation of protein kinase activity by warming prorepressor at 34° for 6 hours (Frame B), was almost completely blocked by hemin (Frame C). When hemin was added after the warming period (Frame D) it had almost no inhibitory effect on the protein kinase activity generated, in-

TABLE 1

Effect of saturating amounts of different HCR preparations on the phosphorylation of IF-1.

Standard protein kinase assay samples (100 µl), containing 50 units of the indicated HCR preparation, were incubated at 34° for 20 minutes. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and stained, scanned, and counted as described in the methods. CPM represent the summed radioactivity associated with the 38K subunit. A blank of 1350 CPM, representing the radioactivity in the 38K region of a control sample, incubated without HCR, has been subtracted.

HCR Preparation	CPM
Crude HCR	9,690
Highly Purified HCR	8,430
NEM Prepared HCR	9,610

dicating that hemin prevents the formation of the protein kinase activity (just as it prevents formation of inhibitory activity) and, at the level used, does not prevent phosphorylation of IF-1. This is in agreement with the fact that the inhibitory activity of HCR, formed after 6 hours at 34°, is insensitive to hemin. It is also consistent with our earlier observations that reversible HCR (formed after brief warming of prorepressor) is inactivated directly by hemin and that hemin does not block its action on protein synthesis (3,21). Hemin has been shown to inhibit the activity of several protein kinases from rabbit reticulocytes (22), but this does not appear to represent the mechanism by which hemin regulates protein synthesis in this system.

Data in Figure 1 indicated that highly purified and NEM prepared HCR had only about one-half as much protein kinase activity (as measured by the phosphorylation of the 38K component of IF-1) as would be expected from their inhibitory activity in the lysate, when compared to crude HCR. The 38K component of IF-1 was labelled to about the same degree by saturating amounts of all three HCR preparations (Table 1), indicating that crude HCR does not contain a separate protein kinase or essential cofactor that is completely absent in the other two preparations. It is possible, however, that highly purified and NEM prepared HCR may have a limiting amount of an essential cofactor or completely lack a component that enhances but is not absolutely essential for HCR activity, and that this cofactor or component is not limiting in crude HCR or the lysate.

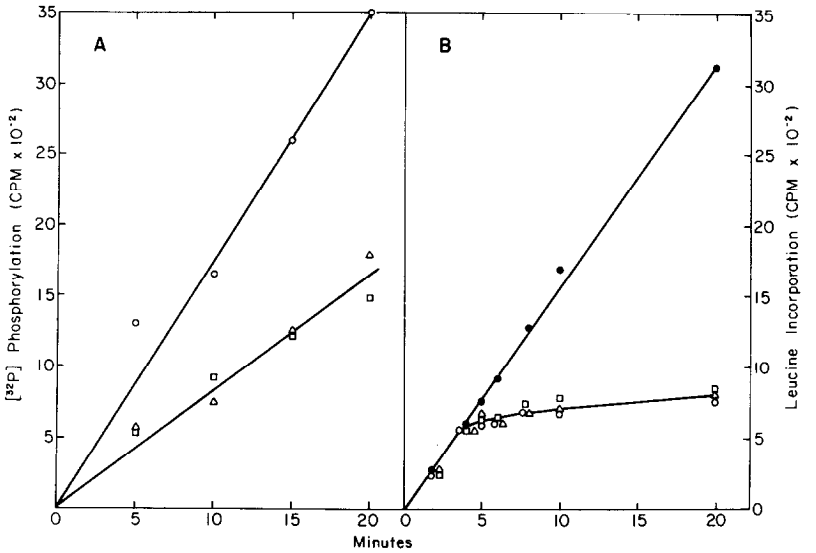

Figure 3. A comparison of the effect of different HCR preparations on the rate of phosphorylation of IF-1 and the rate of protein synthesis in the reticulocyte lysate.

(A) Standard protein kinase assay samples (final volume, 250 µl) contained 13 units of crude (circles), highly purified (triangles), or NEM prepared (squares) HCR. At the indicated times, 50 µl of each reaction mixture were removed and mixed with 10 µl of denaturing solution. Samples were electrophoresed on SDS-polyacrylamide gels, stained, scanned and counted as described in the methods. The ordinate represents the summed radioactivity incorporated into the 38K subunit (minus a corresponding blank value from a sample incubated without HCR). (B) Cell-free protein synthesis samples contained 100 µl of lysate (plus 5 nMoles of hemin), 60 µl of master mix, and 60 µl of Buffer A containing no added HCR (closed circles) or 10 units of crude (open circles), highly purified (triangles), or NEM prepared (squares) HCR. Incubation was at 34°, and at the indicated times, 20 µl were removed for the determination of acid precipitable radioactivity.

The possibility that highly purified and NEM prepared HCR may be unstable when incubated in the protein kinase assay but not in the lysate was tested by studying the time course of the protein kinase reaction for the three HCR preparations. The results (Figure 3, Frame A) demonstrate that the rate of phosphorylation of the 38K component of IF-1 by NEM prepared and highly purified HCR was linear for 20 minutes and was about one-half the rate seen with crude HCR. Thus the difference in protein kinase activity cannot be attributed either to a delay in onset or premature termination of the protein kinase reaction in the case of highly purified or NEM prepared HCR. For comparison, all three HCR preparations had a similar effect on the rate of protein synthesis (Figure 3, Frame B).

TABLE 2

Effect of mixing different HCR preparations on protein kinase activity.

Standard protein kinase assay samples (100 µl), containing the indicated amounts of the indicated HCR preparation, were incubated at 34° for 10 minutes. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and stained, scanned, and counted as described in the methods. Protein kinase activity represents the summed radioactivity incorporated into the 38K subunit (minus a blank value of 1300 CPM obtained in this region when IF-1 was incubated in the absence of HCR).

HCR Preparation	Protein Kinase Activity (CPM)	Calculated Sum ((
Crude HCR (4 units)	3400	-
Highly purified HCR (8 units)	2160	-
NEM prepared HCR (8 units)	2600	-
Crude HCR + highly purified HCR	5150	5560
Crude HCR + NEM prepared HCR	5560	6000
Highly purified HCR + NEM prepared HCR	4680	4760

A separate possibility, that highly purified and NEM prepared HCR may contain a protein kinase inhibitor or phosphatase, not present in crude HCR, that is less effective in the lysate than in the protein kinase assay, was tested by measuring the protein kinase activity of mixtures of the HCR preparations. In all cases (Table 2), the protein kinase activity of a mixture was about equal to the sum of the activities of each preparation individually, suggesting that the difference in protein kinase activity between the HCR preparations is not due to the effect of an inhibitor or phosphatase.

DISCUSSION

Recent findings have suggested that the hemin-controlled translational repressor may act by phosphorylating a subunit of the initiation factor (IF-1) that mediates binding of the initiator t-RNA to the 40 s subunit: (1) HCR appears to act catalytically on the ribosomes (18); (2) a specific protein kinase that phosphorylates the 38,000 molecular weight subunit of IF-1 co-purifies with HCR (16,17); and (3) the action of HCR is dependent upon ATP (23, T. Hunt, personal communication). The results presented here add

additional support to this hypothesis: (1) the prorepressor, which has little inhibitory activity, has little capacity to phosphorylate the 38K component of IF-1; (2) prolonged warming of the prorepressor or brief incubation of the prorepressor with NEM produces the translational inhibitor and the protein kinase that phosphorylates the 38K subunit; (3) hemin prevents the formation of both the inhibitory and protein kinase activities.

We have also found that this specific protein kinase copurifies with HCR. We have noted, however, that highly purified HCR and NEM prepared HCR have only about one-half as much protein kinase activity as would be predicted from their inhibitory activity in the crude lysate, when compared to crude HCR. This difference is seen as a decreased rate of phosphorylation of the 38K component of IF-1 and does not appear due to an inhibitor or phosphatase in the highly purified or NEM prepared HCR samples. Although the reason for this difference is not at present clear, it may be due to a component in the lysate, which enhances or is required for HCR activity that is absent or limiting in highly purified and NEM-induced HCR, but not in crude HCR, preparations.

Using the data in Table 1 as an estimate of the maximal phosphorylation of the 38K subunit of IF-1 by HCR, the gel scans of IF-1 as an estimate that the 38K component represents 8% of the total protein in the factor preparation, a molecular weight for the subunit of 38,000 and a specific activity for the phosphate incorporated of 200 Ci/mole, we have calculated that about 0.8 moles of phosphate are incorporated per mole of IF-1 subunit. Although this figure is approximate, it is consistent with the phosphorylation by HCR of a single site on the 38K subunit.

Although recent findings including those reported here support the concept that HCR inhibits polypeptide chain initiation by phosphorylating IF-1, the precise effect of phosphorylation on the activity of this factor is not at present clear. Preincubation of IF-1 with HCR and ATP under conditions where phosphorylation is maximal does not result in any detectable loss of activity, whether the binding of [^{35}S]Met-tRNA$_f$ to millipore filters or the stimulation of protein synthesis in the lysate is assayed (M. Gross, unpublished). In addition, the stimulation by IF-1 of protein synthesis in the absence of hemin is little affected by as much as 1000 units/ml of crude HCR (M. Gross, manuscript in preparation). One explanation for these findings is that phosphorylation of IF-1 by HCR does not prevent the factor from promoting the formation of 40 s-Met-tRNA$_f$ complexes directly but slows the cycling of the factor from subunit to subunit. This could secondarily inhibit the conversion of 40 s initiation complexes to 80 s complexes as suggested by Balkow et al. (9,10) as well as the formation of more 40 s complexes as suggested by Legon et al. (4). This explanation is also consistent with our

recent observation that IF-1, which promotes the formation of 40 s-Met-tRNA$_f$ complexes, and IF-2, which promotes the formation of 80 s initiation complexes from 40 s complexes, can each partially reverse the inhibitory effect of hemin deprivation or HCR (M. Gross, manuscript submitted). In addition, Cherbas and London have also recently suggested that HCR may act by blocking the recycling of IF-1 (24). HCR, even at great excess, may have little effect on the activity of exogenous IF-1, possibly because the isolated factor may cycle very poorly in the lysate (which could explain why so much factor must be added to stimulate protein synthesis). Other explanations are, of course, also possible, and further investigation will be required to define exactly what effect phosphorylation of IF-1 has on its activity.

One additional consideration is that the lysate must contain a phosphatase that reverses the effect of HCR, since the inhibition of protein synthesis in the absence of hemin can be reversed by hemin alone (3,25,26). Traugh et al. have described a phosphatase from rabbit reticulocytes that removes a phosphate from a site on the 40 s subunit (27). Whether this or some other phosphatase removes the phosphate bound to IF-1 by HCR has yet to be determined.

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