Biochemical Pharmacology, Vol. 37, No. 21, pp. 4089–4093, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

INDUCTION OF THE 32-kD HUMAN STRESS PROTEIN BY AURANOFIN AND RELATED TRIETHYLPHOSPHINE GOLD ANALOGS

MADELYN M. CALTABIANO,* GEORGE POSTE and RUSSELL G. GREIG

Department of Cell Biology, Smith Kline & French Laboratories, Philadelphia, PA 19101; and Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

(*Received* 30 *September* 1987; *accepted* 22 *April* 1988)

Abstract—Challenge of human cells with auranofin, 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*-triethylphosphine gold(I) (Ridaura), a gold-containing compound approved by the FDA for the treatment of rheumatoid arthritis, induces the specific synthesis of a 32-kD stress protein (p32) [Caltabiano *et al.*, *Biochem. biophys. Res. Commun.* **138**, 1074 (1986)]. To establish a structure–activity relationship for this effect, a series of auranofin ligands, gold analogs, and other anti-arthritic agents were examined for their abilities to stimulate p32 synthesis. The results indicate that the gold atom is necessary for enhanced expression of p32. However, the structure of co-ordinated ligands also affected potency, and gold complexes bearing several phosphine or thiosugar groups exhibited the greatest activity. These data indicate that the distinct potencies of auranofin analogs probably reflect their membrane permeability and subsequent delivery of pharmacologically active concentrations of gold to the cytoplasmic compartment.

In response to diverse physical and chemical insults, both procaryotic and eucaryotic cells display a stress response which includes the selective and reversible expression of highly conserved genes that encode for a small number of polypeptides with molecular masses ranging from 22 to 110 kD [1–3]. The enhanced synthesis of these "heat-shock" or "stress" proteins can be elicited by a number of stimuli including hyperthermia [1–6], amino acid analogs [7], heavy metals [8–11], sulfhydryl-reactive agents [12], ethanol [13], ionophores [6], viral infection [14], and glucose deprivation [6]. The precise function(s) of stress proteins has not been established but their ubiquitous distribution suggests that they may play a role in protection against environmental insults [1–3, 15].

Previously, we described the specific induction of a 32-kD stress protein (p32) in both normal and neoplastic mammalian cells by certain heavy metals and thiol-reactive agents [16, 17]. The most potent inducer of p32 synthesis is auranofin, a sulfhydryl-reactive gold compound used in the treatment of rheumatoid arthritis [17]. The main goal of the present study was to investigate which regions of the auranofin molecule were necessary for, or influential in, stimulating the expression of p32.

MATERIALS AND METHODS

Agents. Ligands and gold salts were obtained from Smith Kline & French Laboratories (Philadelphia, PA). All other compounds were purchased from the Sigma Chemical Co. (St. Louis, MO). Agents insoluble in water were freshly prepared in dimethyl sulfoxide or ethanol at a final solvent concentration of 1%.

Cell culture, stress conditions, and metabolic labeling. A375 human melanoma cells were propagated in monolayer culture as described previously [16]. Cells were seeded into plastic dishes and incubated overnight at 37°. To study the kinetics of p32 induction, confluent cultures were rinsed with phosphate-buffered saline (GIBCO), challenged with auranofin (0.5 μM) in serum-free Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) for various times at 37°, and labeled with 100 μCi/ml of [^{35}S]methionine (L-[^{35}S]methionine, >800 Ci/mmol; Amersham Corp., Arlington Heights, IL) in DMEM lacking methionine for the last hour of stress. For dose–response studies, cells were challenged with test agents in serum-free DMEM for 6 hr at 37° and radiolabeled with 100 μCi/ml of [^{35}S]methionine for the last 4 hr of stress.

Gel electrophoresis and fluorography. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously [16]. Briefly, cell monolayers were rinsed with phosphate-buffered saline, solubilized on ice for 20 min in 10 mM Tris buffer, pH 7.6, containing 1% Nonidet P-40, 0.1% SDS, 0.15 M NaCl, 1% Trayslol, and 1 mM phenylmethylsulfonyl fluoride and scraped from the culture vessels with a rubber policeman. Extracts were centrifuged in an Eppendorf microcentrifuge at 4° for 20 min, and the supernatant fractions were mixed with an equal volume of sample buffer and boiled for 3 min. Equal amounts of trichloroacetic acid (TCA)-insoluble radioactivity were analyzed on SDS-PAGE using the discontinuous buffer system of Laemmli

* Address correspondence to: Madelyn M. Caltabiano, Ph.D., Department of Cell Biology (L-109), Smith Kline & French Laboratories, 1500 Spring Garden St., Philadelphia, PA 19101.

[18] with a 4.5% acrylamide stacking gel and a 12.5% acrylamide resolving gel. TCA-insoluble radioactivity was estimated as described [16], and molecular weight calibrations were determined using low molecular weight protein standards (Pharmacia, Piscataway, NJ). Following electrophoresis, gels were fixed, stained with Coomassie Brilliant Blue G-250, impregnated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed to preflashed KODAK X-OMAT AR X-ray film (Fotodyne, New Berlin, WI).

Synthesis of p32 was quantified by densitometric analysis of fluorograms using a Beckman DU-8 spectrophotometer. Since actin synthesis remained constant under the conditions used, the ratio of actin to p32 expression was a convenient measure of p32 induction. In control, unstressed cells, this ratio (R) was arbitrarily set at 1.0. Thus, R values greater than unity indicate enhanced p32 synthesis, whereas ratios less than 1.0 reflect suppressed expression of p32.

RESULTS

Kinetics of p32 induction. To optimize conditions for the induction of p32, A375 melanoma cells were stressed with auranofin (0.5 μM) for different times. The major alteration in protein synthesis was enhanced production of p32 (Fig. 1). Elevated synthesis was detected 4 hr after auranofin challenge and peaked 2 hr later (Fig. 1). In all of the dose–response studies outlined below, therefore, p32 expression was evaluated 6 hr after addition of the test agent.

Structure–activity relationships. To establish a

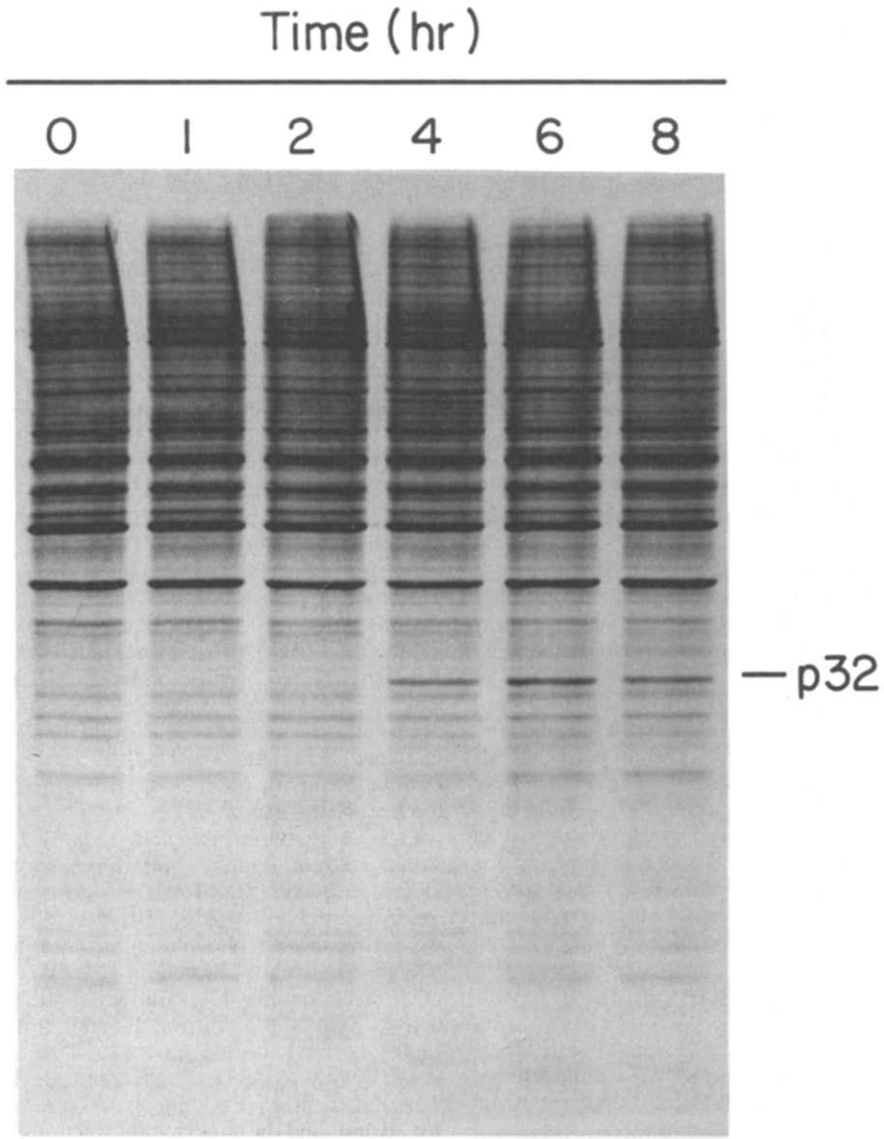


Fig. 1. Kinetics of p32 induction. Monolayer cultures of A375 melanoma cells were challenged with auranofin (0.5 μM) for the indicated times and radiolabeled with [^{35}S]methionine (100 μCi/ml) for the last hour of stress. Cell extracts containing equivalent amounts of TCA-insoluble radioactivity (100,000 cpm) were analyzed by SDS-PAGE and fluorography as described in Materials and Methods.

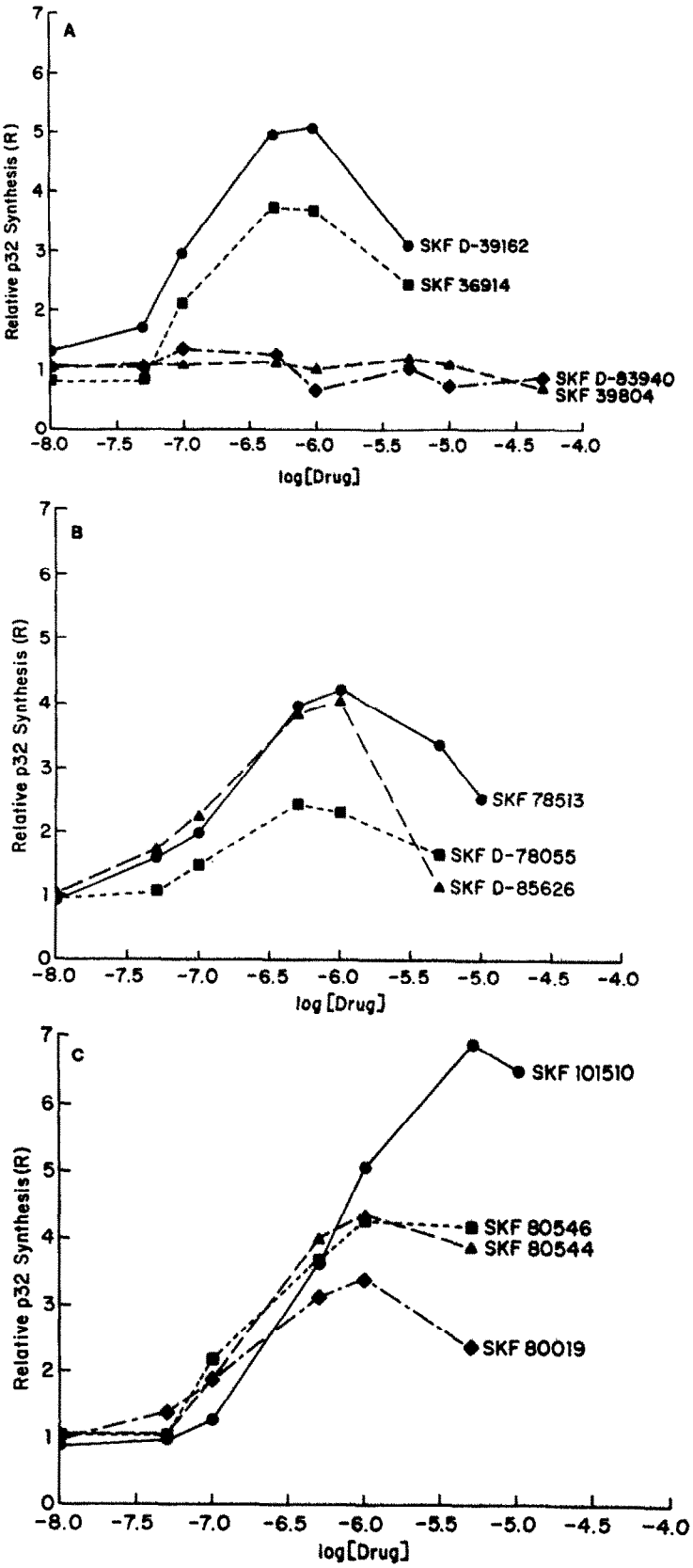


Fig. 2. Induction of p32 synthesis by: (A) auranofin and its ligands; (B) phosphine-co-ordinated gold thiosugar complexes; and (C) gold–phosphine compounds. Relative p32 synthesis (R) was estimated as described in Materials and Methods.

structure–activity relationship for the induction of p32 by auranofin, several auranofin ligands, gold analogs and other anti-arthritic agents were examined for their abilities to enhance p32 synthesis in A375 melanoma cells. Figure 2 shows the dose–response profiles for induction of p32 by auranofin (SKF D-39162) and its ligands. Detectable p32 synthesis required a minimal auranofin concentration of 0.05 μM ($R_{0.05\mu M} = 1.7$), while maximal production occurred at 0.5 to 1 μM ($R_{1\mu M} = 5.06$) (Fig. 2A). In contrast, of the three auranofin ligands tested only the triethylphosphine gold salt (SKF 36914) induced p32 expression, but this compound was considerably less potent ($R_{1\mu M} = 3.67$) than auranofin. Both auranofin and SKF 36914 caused diminished expression of p32 at higher concentrations (greater than 1 μM) and induced considerable toxicity at doses above 10 μM. Neither triethylphosphine oxide (SKF 39804) nor tetra-acetyl-thioglucopyranose (SKF D-83940) induced p32 synthesis (Fig. 2A).

Like auranofin, three related phosphine gold thiosugars (SKF D-85626, D-78055, and 78513) elicited p32 expression at low concentrations (0.05 to 1.0 μM) (Fig. 2B). However, these agents differed markedly in their abilities to induce maximum levels of p32 synthesis. The bis-triethylphosphine and galactose analogs (SKF D-85626 and 78513 respectively) induced considerable p32 expression ($R_{1\mu M} = 4.07$ and 4.25 respectively), but the triphenylphosphine analog (SKF D-78055) was significantly less potent ($R_{1\mu M} = 2.32$). Again, for each compound tested, significant cytotoxicity was observed at doses above 10 μM.

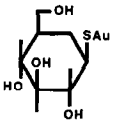
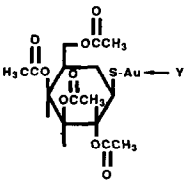
Several other phosphine gold compounds were examined, and these agents fell into two major categories. Compounds in the first class, including tris-triethylphosphine sulfonium (SKF 101510), bis-triethylphosphine (SKF 80544), bis-trimethylphosphine (SKF 80546), and methyl-triethylphosphine (SKF 80019) gold salts, induced a marked increase in p32 expression ($R_{1\mu M} \geq 3.4$) (Fig. 2C). In contrast, a second group of agents stimulated lower levels of p32 synthesis ($R_{1\mu M} = \leq 2.4$). This group included triphenylphosphine (SKF 40738), trimethylphosphine (SKF 40337), and cyanotriethylphosphine (SKF 60817) gold (data not shown).

Of the non-phosphine gold compounds studied, diethylsulfide gold (SKF 79697) and pentamethylene sulfide gold (SKF 79521) induced intermediate levels of p32 synthesis ($R_{1\mu M} = 2$–3). Gold sodium thiomalate (SKF 34790Z_2) and gold thioglucose (SKF 10056), injectable gold compounds currently used in the treatment of rheumatoid arthritis, failed to elicit significant expression of p32 ($R_{1\mu M} < 1.0$) (data not shown).

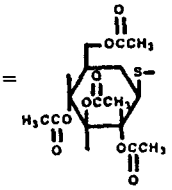
The data are summarized in Table 1 which classifies each compound according to its ability to induce p32. Class I agents were weak or ineffective inducers ($R_{1\mu M} \leq 1.0$), whereas Class III compounds were the most active ($R_{1\mu M} \geq 4.0$). Agents with intermediate potency ($R_{1\mu M} > 1.5 \leq 3.7$) are listed under Class II.

Induction of p32 synthesis by anti-arthritic non-gold compounds. A number of anti-arthritic agents used in routine therapy were also evaluated for their abilities to induce p32. These included: non-steroidal anti-inflammatory drugs (NSAIDS) (acetylsalicylic

Table 1. Induction of p32 and inhibition of protein synthesis by different gold compounds

Compound (SKF)	Structure	p32 Synthesis[a] ($R_{1\mu M}$)
Class I		
D-83940	X[b]H	0.65
10056		0.88
39804	O=Y[c]	1.00
34790Z_2	AuSCHCOOH | CH_2COOH	1.08
Class II		
60817	CNAu←Y	1.56
40337	ClAu←$P(CH_3)_3$	2.24
D-78055	XAu←P()3	2.32
79521	ClAu←S 	2.36
40738	ClAu←P()3	2.39
79697	ClAu←$S(CH_2CH_3)_2$	3.10
80019	CH_3Au←Y	3.44
36914	ClAu←Y	3.67
Class III		
D-85626	X(Au←Y)$_2^+Cl^-$	4.07
78513		4.25
80546	$Cl^-Au^+[P(CH_3)_3]_2$	4.31
80544	$Cl^-Au^+(Y)_2$	4.41
D-39162	XAu←Y	5.06
101510	$S^+NO_3^-$(Au←Y)3	5.10

[a] R is the ratio of p32/actin synthesis as defined in Materials and Methods.

[b] X = 

[c] Y = $P(CH_2CH_3)_3$.

acid, 10^{-7}–5×10^{-3} M; indomethacin, 10^{-7}–5×10^{-4} M; and phenylbutazone, 10^{-7}–5×10^{-3} M); corticosteroids (dexamethasone, 10^{-7}–5×10^{-3} M; and hydrocortisone, 10^{-7}–5×10^{-4} M); disease-modifying anti-rheumatoid drugs (DMARDS) (penicillamine, 10^{-8}–5×10^{-3} M; and chloroquine, 10^{-8}–5×10^{-5} M); immunomodulators (levamisole, 10^{-7}–5×10^{-3} M); and immunosuppressives (azathioprine, 10^{-7}–5×10^{-4} M; and methotrexate, 10^{-7}–5×10^{-3} M). All agents were inactive under the conditions used, though production of a 70-kD stress protein was observed in cells challenged with high doses of azathioprine, levamisole or phenylbutazone (data not shown).

DISCUSSION

In previous investigations we have described the specific induction of a 32-kD protein (p32) in both normal and neoplastic human cells stressed with heavy metals or sulfhydryl-reactive agents [16, 17]. Auranofin, a gold-containing, sulfhydryl-reactive drug approved by the FDA for the treatment of rheumatoid arthritis, is particularly active and induces p32 synthesis at pharmacologic concentrations (10^{-8} to 10^{-6} M) [17]. In contrast, gold chloride induces only minimal expression of p32 even when used at toxic doses (10^{-4} M) [16]. The objectives of the present study were to establish whether the gold atom was essential for auranofin's activity and, if so, to evaluate the influence of different ligands on the ability of gold complexes to enhance expression of p32. While gold-containing analogs of auranofin displayed a spectrum of potencies in stimulating p32 synthesis, structures lacking the gold atom, including the non-gold auranofin ligands [triethylphosphine oxide (SKF 39804) and tetra-acetyl-thioglucopyranose (SKF D-83940)] and several non-metallic anti-arthritic agents (e.g. non-steroidal anti-inflammatory drugs, corticosteroids and disease-modifying anti-rheumatoid drugs) were consistently inactive. These data, coupled with previous observations [16, 17], confirm that heavy metals, including gold, play an important role in regulating p32 expression.

However, it was also clear that the ligands attached to the co-ordinating gold atom were critical in determining the ability of a compound to stimulate p32 expression. For example, agents containing two or three large hydrophobic groups such as triethyl-(methyl)phosphine and tetra-acetyl-thioglucose (galactose) induced peak p32 expression. Compounds belonging to this category include auranofin (SKF D-39162), the galactose analog (SKF 78513), and the bis (SKF D-85626, 80546 and 80544) and tris (SKF 101510) compounds. One possible explanation for these structure–activity relationships is that the hydrophobic groups facilitate permeability of the gold compounds through the plasma membrane. The higher intracellular concentration of gold thus attained is presumably responsible for stimulating elevated expression of p32. This hypothesis is consistent with the model of auranofin membrane transport and intracellular distribution proposed recently by Synder *et al.* [19]. In addition, the inability of gold chloride, gold thioglucose (SKF 10056) and gold thiomalate (SKF 34790Z_2) to induce p32 synthesis provides further support for this proposal since these compounds are significantly less lipophilic, partitioning almost exclusively into water as compared to octanol, than auranofin and its phosphine analogs [20].

Collectively, these results indicate that the gold atom of auranofin is essential for stimulation of p32 production. However, structural modifications of the gold-complexed ligands altered significantly the ability to induce p32 synthesis. In particular, gold complexes with large hydrophobic ligands were significantly more active compared to compounds bearing hydrophilic moieties. Whether p32 expression plays a role in mediating the pharmacology of anti-arthritic gold compounds has yet to be addressed, but it is interesting to speculate that *in vitro* synthesis of p32 may provide a convenient system for screening novel, gold-based, anti-arthritic agents more potent than auranofin.

Acknowledgements—We thank Dr. Christopher Mirabelli for comments on the manuscript, Jo Anne Mackey for excellent secretarial assistance, and Scot Gerding and Ray Gues for professional photographic services.

REFERENCES

1. M. J. Schlesinger, M. Ashburner and A. Tissieres (Eds.), *Heat Shock: From Bacteria to Man.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
2. L. Nover, D. Hellmund, D. Neumann, K-D. Scharf and E. Serfling, *Biol. Zbl.* **103**, 357 (1984).
3. B. G. Atkinson and D. B. Walden (Eds.), *Changes in Eukaryotic Gene Expression in Response to Environmental Stress.* Academic Press, New York (1985).
4. P. M. Kelley and M. J. Schlesinger, *Cell* **15**, 1277 (1978).
5. R. Voellmy, P. Bromley and H. P. Kocher, *J. biol. Chem.* **258**, 3516 (1983).
6. W. J. Welch, J. I. Garrels, G. P. Thomas, J. J-C. Lin and J. R. Feramisco, *J. biol. Chem.* **258**, 7102 (1983).
7. G. C. Li and A. Laszlo, *J. cell. Physiol.* **122**, 91 (1985).
8. D. Johnston, H. Oppermann, J. Jackson and W. Levinson, *J. biol. Chem.* **255**, 6975 (1985).
9. Y-J. Kim, J. Shuman, M. Sette and A. Przybyla, *J. Cell Biol.* **96**, 393 (1983).
10. J. J. Heikkila, G. A. Schultz, K. Iatrou and L. Gedamu, *J. biol. Chem.* **257**, 12000 (1982).
11. S. A. Whelan and L. E. Hightower, *J. cell. Physiol.* **122**, 205 (1985).
12. W. Levinson, H. Oppermann and J. Jackson, *Biochim. biophys. Acta* **606**, 170 (1980).
13. G. C. Li, *J. cell. Physiol.* **115**, 116 (1983).
14. E. W. Khandjian and H. Turler, *Molec. cell. Biol.* **3**, 1 (1983).
15. G. P. Thomas, W. J. Welch, M. B. Mathews and J. R. Feramisco, *Cold Spring Harbor Symp. Quant. Biol.* **45**, 985 (1982).
16. M. M. Caltabiano, T. P. Koestler, G. Poste and R. G. Greig, *J. biol. Chem.* **261**, 13381 (1986).
17. M. M. Caltabiano, T. P. Koestler, G. Poste and R. G. Greig, *Biochem. biophys. Res. Commun.* **138**, 1074 (1986).
18. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
19. R. M. Synder, C. K. Mirabelli and S. T. Crooke, *Biochem. Pharmac.* **35**, 923 (1986).
20. B. M. Sutton, J. C. Hempel and D. T. Hill, in *Current Clinical Practice Series 7* (Eds. H. A. Capell, D. S. Cole, K. K. Manghani and R. W. Morris), p. 6. Excerpta Medica, Amsterdam (1983).