Blotting and band-shifting: techniques for studying protein–protein interactions

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The type II cAMP-dependent protein kinase (PKA) is localized in certain cellular compartments through association with specific A-kinase anchoring proteins (AKAPs). A variety of blotting and electrophoresis techniques have been developed to study the protein–protein interactions that occur between the regulatory (R) subunit of PKA and AKAPs. These methods have also been used for a variety of purposes such as detecting calmodulin-binding proteins, comparing wild-type- and mutant-form binding affinities and estimating the molecular weight of multiprotein complexes.

THE SUBCELLULAR LOCATION of the type II cAMP-dependent protein kinase (PKA) is maintained through interaction between the regulatory subunit of the kinase (RII) and specific anchoring proteins (for review see Ref. 1). Subcellular fractionation and immunolocalization techniques have demonstrated that a significant proportion of the type II PKA is associated with the plasma membrane, cytoskeletal components, secretory granules or the nuclear membranes[2–7]. More recently, Rubin, Erlichman and others have characterized a family of proteins which co-purify with RII after affinity chromatography on cAMP–sepharose[8–10]. Rubin and colleagues have named these molecules A-kinase anchoring proteins (AKAPs) because they are capable of tethering the type II PKA to specific subcellular sites, presumably co-localizing the kinase close to physiological substrates[11]. This article describes three techniques, listed below, which have been used extensively to study the protein–protein interactions between the type II PKA and AKAPs.

(1) A modified western blot procedure using radiolabeled RII as a probe, called the RII-overlay technique, which has been successfully used by several laboratories to identify numerous AKAPs.

(2) An expression cloning strategy using radiolabeled RII as a probe, resulting in the isolation of several cDNAs encoding AKAPs[12–15].

(3) Gel electrophoresis under non-denaturing conditions, called 'band-shift' analysis, used to demonstrate the formation of multiprotein complexes between certain AKAPs and the PKA holoenzyme (R2C2)[14,16]. This technique has also been used to determine the relative binding affinities of mutant AKAPs.

While the examples in this article focus on the use of protein blotting and band-shift methods to study PKA anchoring, these techniques can easily be adapted to study multiprotein complexes in a variety of experimental systems.

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The RII-overlay technique

Detailed study of PKA anchoring was made possible by the original observation of Lohmann and colleagues[10] that many, though most likely not all, AKAPs retain their ability to bind RII after transfer to nitrocellulose. As a result, an overlay technique has been developed which is essentially a modification of the western blot procedure[17,18]. Protein samples are separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose by standard electrotransfer techniques[16]. The immobilized protein is partially renatured by incubation in a blocking solution of powdered milk before reaction with ^{32}P-labeled RII probe. After extensive washing to remove uncomplexed RII, AKAPs are detected by autoradiography. A five- to tenfold increase in sensitivity can be achieved if bound RII is detected immunologically with anti-RII-antisera and ^{125}I-labeled protein A. As can be seen in Fig. 1a, a survey of nine bovine tissue homogenates detected numerous RII-binding bands ranging in size from 34 to 300 kDa. Greater separation of these proteins is achieved if a two-dimensional RII-overlay procedure is used (Fig. 1b). Crude extracts from various tissues are separated by isoelectric focusing (IEF) in the first dimension and by SDS-PAGE in the second dimension. After electrotransfer, the standard overlay procedure is performed. Surprisingly, both overlay procedures are highly specific for detecting AKAPs. No RII binding is detected if RIIΔ1–23, a deletion mutant unable to interact with AKAPs, is used as a probe in overlay experiments[19].

Expression cloning with RII as a probe

An elegant extension of the overlay technique, first used by Rubin and colleagues, is the use of RII to screen cDNA expression libraries. This protocol is an adaptation of the antibody screening method of Helfman[20], but uses ^{32}P-labeled RII as a probe. Using this strategy Rubin and colleagues isolated cDNAs for two anchoring proteins, AKAP 75 and AKAP 150 (Refs 12,13). More recently, this procedure has been modified by preabsorbing filters with a tenfold molar excess of RIIΔ1–23, a mutant that does not bind anchoring proteins. This step cuts down on the non-specific interaction of the probe with bacterial cell debris and prevents detection of cDNAs encoding the C subunit of PKA which bind RIIΔ1–23 with high affinity. To date, a total of eight AKAP cDNAs have been identified and characterized using this screening method[12–15].

Band-shift analysis

Although overlay methods are effective for screening and identifying proteins that bind RII, they cannot quantitate binding or determine the molecular weight of a multiprotein complex. Additionally, the harsh conditions used to separate and transfer proteins to nitrocellulose may eliminate certain AKAPs from solid-phase interaction with RII. To overcome some of these limitations, we have used a band-shift technique that examines protein–protein

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interactions under non-denaturing conditions[14,16]. The method takes advantage of the observation that a protein complex migrates on a native polyacrylamide gel with a different motility than its individual components. Proteins are preincubated in binding buffer to allow complex formation. After dilution with sample buffer, free and complexed proteins are separated by electrophoresis. Protein bands are detected using either Coomassie Blue or autoradiography. The band-shift technique was used to detect the formation of the type II PKA holoenzyme complex (Fig. 2a) and its interaction with recombinant fragments of two AKAPs, microtubule-associated protein (MAP2) and Ht 31. This technique demonstrates that AKAPs bind to the RII subunit, whether or not it is part of the PKA holoenzyme.

Another application of the band-shift is to compare the relative binding affinities of AKAPs. Figure 2b compares the binding properties of wild-type Ht 31 to a mutant with reduced affinity for RII. The binding affinities of several Ht 31 mutants, containing amino acid substitutions within the RII-binding domain, have been assessed using this method. The sensitivity of this technique is limited by the high concentration of protein required for detection of complexes and because equilibrium conditions are not maintained during electrophoresis. However, greater sensitivity can be achieved by utilizing a competitive binding assay in conjunction with the band-shift technique. A peptide encompassing the RII-binding domain of Ht 31, a human thyroid AKAP, has been synthesized. Binding of radiolabeled peptide to RIIα can be detected by auto-

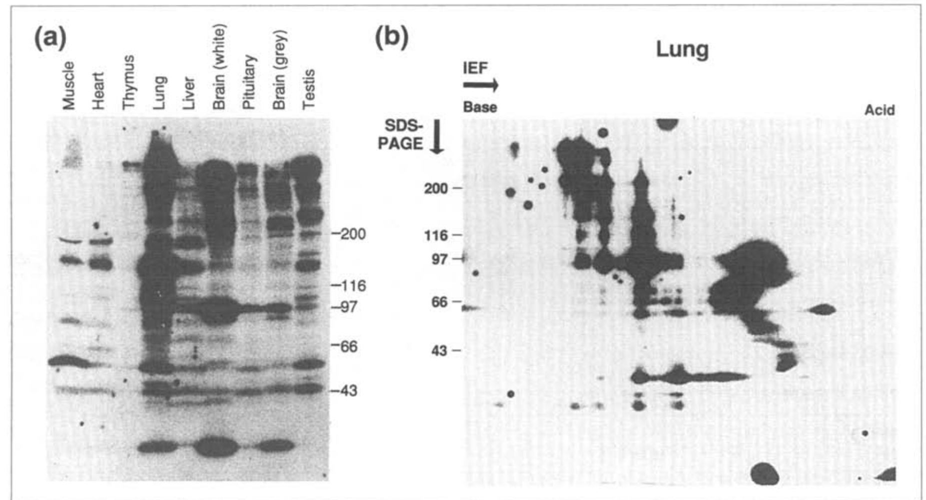

Figure 1

RII overlays. **(a)** Tissue survey of bovine RII-anchoring proteins. Crude protein extracts from nine bovine tissues were analysed by RII overlay for anchoring proteins. Protein samples (100 μg) were separated by electrophoresis on a 4–12% SDS polyacrylamide gradient gel and transferred to immobilon. RII-binding bands were detected by autoradiography after incubation of the blot with ^{32}P-labeled RIIα. Tissue sources are marked above each lane. (Reproduced with permission of *J. Biol. Chem.*) **(b)** Protein extract from bovine lung was separated by isoelectric focusing in the first dimension and electrophoresis on 4–12% gradient SDS-PAGE gels before transfer to immobilon. RII-anchoring proteins were detected by autoradiography after incubation of the blot with ^{32}P-labeled RIIα. The direction of IEF and molecular weight markers are indicated on the sides of each overlay.

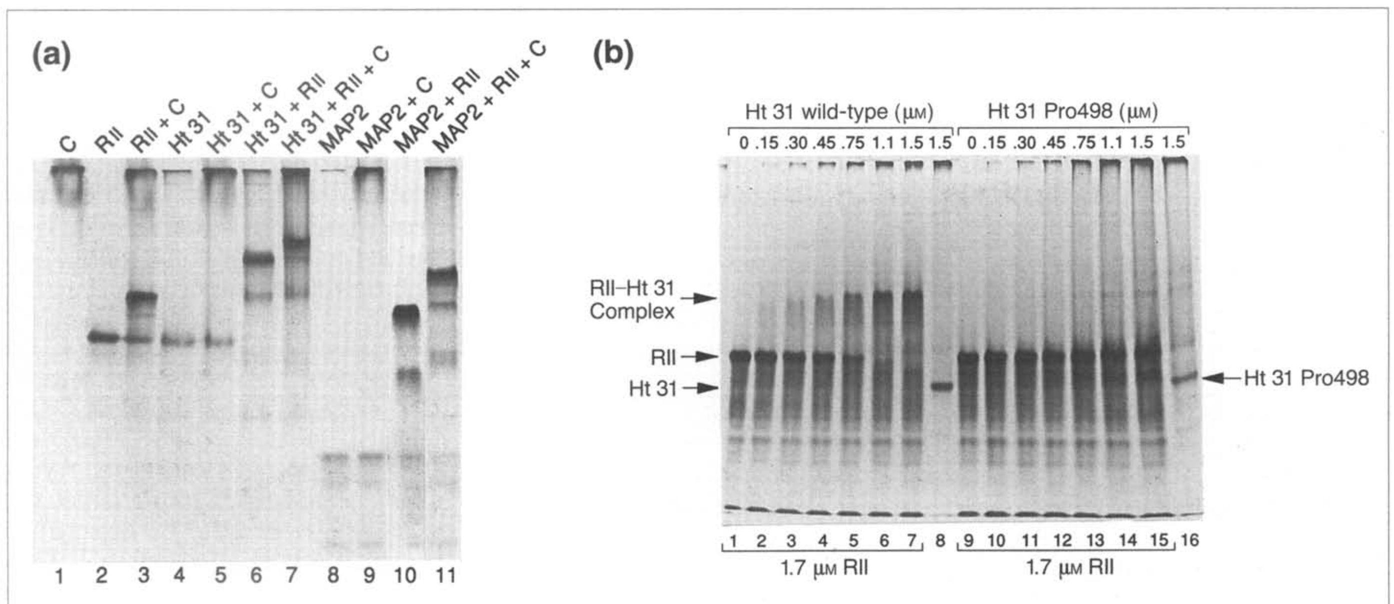

Figure 2

Binding of AKAPs to RIIα and type II PKA holoenzyme under non-denaturing conditions. **(a)** The ability of Ht 31 (418–736) and MAP2 (1–154) to interact with RIIα or the type II PKA holoenzyme was determined by band-shift assay. The migration rate of the proteins or protein complexes is based on size and charge. Complex formation produces a band with a migration rate distinct from any of the components. Various combinations of proteins (5 μg each) were mixed together for 1 h at 22°C before electrophoresing on a 6% (w/v) polyacrylamide gel, and staining with Coomassie Brilliant Blue. Lane 1 contains C subunit (the C subunit is basic and migrates to the top of the lane); lane 2, RIIα; lane 3, RIIα + C (type II PKA holoenzyme); lane 4, Ht 31; lane 5, Ht 31 + C; lane 6, Ht 31 + RIIα; lane 7, Ht 31 + type II PKA holoenzyme; lane 8, MAP2; lane 9, MAP2 + C; lane 10, MAP2 + RIIα; lane 11, MAP2 + type II PKA holoenzyme. (Reproduced with permission of *J. Biol. Chem.*) **(b)** Samples of RII (1.7 μM) and various concentrations of recombinant Ht 31 (wild-type) or Ht 31 (Pro498) were incubated and separated by non-denaturing gel electrophoresis. Free RII, Ht 31 and the RII–Ht 31 complex are indicated.

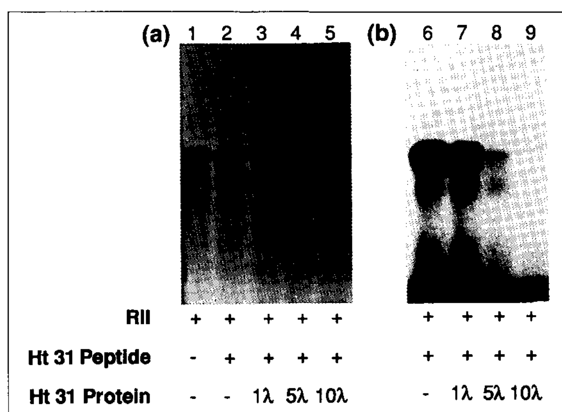

Figure 3
Band-shift analysis of RIIa/Ht 31 peptide–peptide interaction and competition with Ht 31 protein. ^{125}I-labeled Ht 31 (493–515) peptide (4.2 ng μl^{-1}) and RII (4.2 μg μl^{-1}) were incubated for 1 h at 22°C. Samples were incubated further with various concentrations of Ht 31 protein (0.5 μg μl^{-1}) and separated by non-denaturing electrophoresis. Gels were analysed **(a)** by Coomassie Blue staining and **(b)** by autoradiography. The reaction mixtures are indicated below each lane. (Reproduced with permission of *J. Biol. Chem.*)

radiography after electrophoresis on native gels (Fig. 3). Addition of wild-type or mutant anchoring proteins to the reaction mixture displaces the peptide from interaction with RII in a dose-dependent manner. The relative binding affinities can be calculated by determining the concentration of anchoring protein needed to displace 50% of the peptide from RII.

The application of these techniques to study PKA anchoring

Protein blotting and band-shift techniques have been successfully used by various laboratories to monitor the protein–protein interactions involved in PKA anchoring[8–15,19,21]. Cytoskeletal localization of PKA has been shown to occur through association of RII with MAP2. A family of RII deletion mutants expressed in *E. coli* were tested for RII-binding activity using the overlay procedure with immobilized MAP2 or AKAP 75 as a substrate. Data suggest that MAP2 interacts only with the dimerized RII, but that regions distal to the dimerization domain are also required for binding[19,21]. These results have led us to propose a model for PKA anchoring, presented in Fig. 4. This model predicts that all AKAPs bind to the same or overlapping sites on RII and are therefore likely to contain a similar RII-binding motif. In an attempt to identify this RII-binding motif, several cDNAs encoding AKAPs were isolated by expression cloning with RII as a probe[12–15]. Although no primary structure motif was evident, computer-aided analysis of secondary structure identified common regions of 14 residues which displayed high probabilities of forming amphipathic helices[14]. Band-shift analysis was used to show that mutations designed to disrupt secondary structure within the putative helix region of Ht 31, a human AKAP, reduced RII-binding dramatically[14]. Specifically, proline was substituted within the helix region of Ht 31 and reduced RII binding, as assessed by band-shift analysis. In contrast, substitution of proline at a site downstream of the putative helix had no effect on RII-binding. Combined, these data suggest that AKAPs may interact with RII via an acidic amphipathic helix motif (Fig. 4). Recently, this hypothesis has been supported by additional evidence: a peptide encompassing the amphipathic helix region of Ht 31 adopts an α-helical conformation as assessed by circular dichroism and the same peptide binds RII or the type II PKA with nanomolar affinity[15].

Conclusions and perspectives

The techniques described in this article are simple yet powerful methods that should be generally applicable to many other systems. The advantages of the overlay method are that it requires small quantities of probe (1 μg) and is capable of identifying binding proteins with high specificity. In fact, partially purified extracts enriched with the protein probe can be used. Other examples of this technique include the use of radiolabeled calmodulin as a probe for detecting calmodulin-binding proteins[22]. Biotinylated calmodulin also retains its ability to bind to immobilized proteins and is detectable using streptavadin conjugated alkaline phosphatase. This provides a highly sensitive non-radioactive technique which can detect as little as 10 ng of calmodulin-binding protein[23]. Protein kinase C (PKC) has also been shown to bind immobilized cytoskeletal components in the presence of a phosphatidylserine[24,25].

One limitation of the overlay and expression cloning techniques is the harsh treatments employed to separate and immobilize the proteins. Consequently, protein–protein interactions that occur through contact at several sites on the surface of the target protein are less likely to be detected by overlay. For this reason, only about 50% of calmodulin-binding proteins can be detected by overlay methods. Likewise, the association of the regulatory (R) and catalytic (C) subunits in the PKA holoenzyme (R2C2) is not detected in RII overlays[10,15]. Therefore, a determining factor in the success of overlays must be the extent to which a target protein renatures when immobilized on nitrocellulose. Renaturation of immobilized proteins is different for each

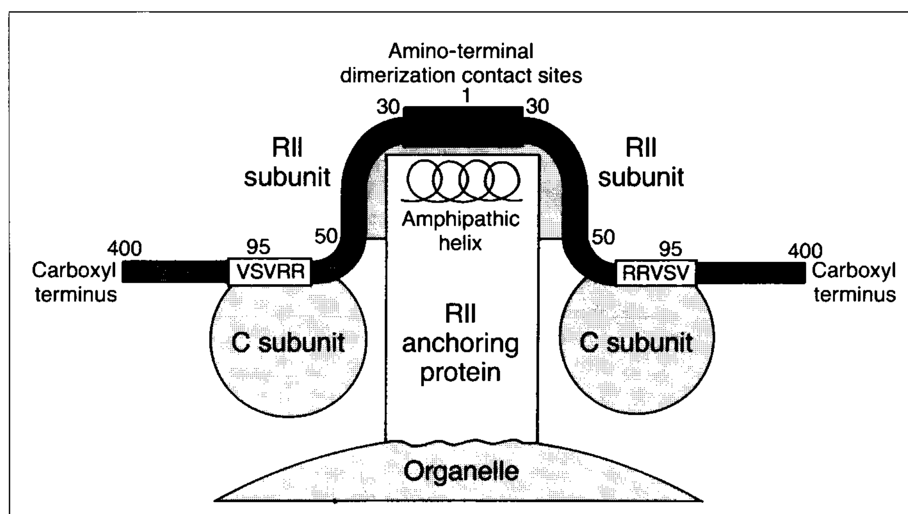

Figure 4
Model for the topology of the anchored type II PKA holoenzyme. This schematic represents the summary of data from many investigators showing how the first 50 residues of murine RIIα could interact with an amphipathic helix on a RII-anchoring protein and thereby target the type II PKA holoenzyme to specific subcellular locations.

molecule and consequently very little data is available on this subject. Renaturation of some immobilized proteins is favored if they are first completely denatured by incubation in solutions of 8 M urea or 6 M guanidine[26]. Denaturation–renaturation cycles have enhanced detection of transcription factor cDNAs from expression libraries screened with enhancer element probes[27]. It is possible that a similar procedure would also improve detection by other protein probes.

Although band-shift analysis requires greater amounts of purified protein (2–5 μg per lane), it provides a more sophisticated analysis of protein–protein interactions. This technique can be used to demonstrate the formation of multiprotein complexes, compare the binding affinities of wild-type and mutant proteins, and as a competition assay to determine the binding affinity of various proteins. The versatility of the band-shift analysis is demonstrated by its use as a method to measure the molecular weight of a multiprotein complex. Protein complexes are analysed on series of non-denaturing gels at increasing concentrations of acrylamide (4–12%). The log of R_f is plotted against the acrylamide concentration and the slope is calculated. Comparison of this value to the slopes of standard proteins allows extrapolation of the molecular weight[16,28]. This can be used to determine the number or stoichiometry of the subunits in a multiprotein complex.

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References

1 Scott, J. D. (1991) *Pharmacol. Ther.* 50, 123–145
2 Hofmann, F., Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1975) *J. Biol. Chem.* 250, 7795–7801
3 Corbin, J. D., Keely, S. L. and Park, C. R. (1975) *J. Biol. Chem.* 250, 218–225
4 Corbin, J. D., Sugden, P. H., Lincoln, T. M. and Keely, S. L. (1977) *J. Biol. Chem.* 252, 3845–3861
5 Rubin, C. S., Erlichman, J. and Rosen, O. R. (1972) *J. Biol. Chem.* 247, 6135–6139
6 Stein, J. C., Farooq, M., Norton, W. T. and Rubin, C. S. (1987) *J. Biol. Chem.* 262, 3002–3006
7 Rubin, C. S. *et al.* (1979) *J. Biol. Chem.* 254, 3797–3805
8 Sarkar, D., Erlichman, J. and Rubin, C. S. (1984) *J. Biol. Chem.* 259, 9840–9846
9 Leiser, M., Rubin, C. S. and Erlichman, J. (1986) *J. Biol. Chem.* 261, 1904–1908
10 Lohmann, S. M., DeCamilli, P., Einig, I. and Walter, U. (1984) *Proc. Natl Acad. Sci. USA* 81, 6723–6727
11 Hirsch, A. H. *et al.* (1992) *J. Biol. Chem.* 267, 2131–2134
12 Bregmann, D. B., Bhattacharyya, N. and Rubin, C. S. (1989) *J. Biol. Chem.* 264, 4648–4656
13 Bergmann, D. B., Hirsch, A. H. and Rubin, C. S. (1991) *J. Biol. Chem.* 266, 7207–7213
14 Carr, D. W. *et al.* (1991) *J. Biol. Chem.* 266, 14188–14192
15 Carr, D. W. *et al. J. Biol. Chem.* (in press)
16 Bollag, D. M. and Edelstein, S. J. (1991) in *Protein Methods*, pp. 143–160, Wiley-Liss
17 Towbin, H., Staehlin, T. and Gordon, J. (1979) *Proc. Natl Acad. Sci. USA* 76, 4350–4354
18 Wade, D. P. and Soutar, A. K. (1989) in *Protein Function, A Practical Approach* (Creighton, T. E., ed.), pp. 55–76, IRL Press
19 Scott, J. D. *et al.* (1990) *J. Biol. Chem.* 265, 21561–21566
20 Helfman, D. M. *et al.* (1983) *Proc. Natl Acad. Sci. USA* 80, 31–35
21 Luo, Z., Shafit-Zargado, B. and Erlichman, J. (1991) *J. Biol. Chem.* 265, 21804–21810
22 Lottenberg, R., Christensen, U., Jackson, C. M. and Coleman, P. L. (1981) *Meth. Enzymol.* 80, 341–361
23 Stofko-Hahn, R. E., Carr, D. W. and Scott, J. D. *FEBS Lett.* (in press)
24 Wolf, M. and Sahyoun, N. (1986) *J. Biol. Chem.* 261, 13327–13332
25 Mochly-Rosen, D., Khaner, H. and Lopez, J. (1991) *Proc. Natl Acad. Sci. USA* 88, 3997–4000
26 Vinson, C. R. *et al.* (1988) *Genes Dev.* 2, 801–806
27 Singh, H., LeBowitz, J. H., Baldwin, A. S., Jr and Sharp, P. A. (1988) *Cell* 52, 415–423
28 Hedrick, J. L. and Smith, A. J. (1968) *Arch. Biochem. Biophys.* 126, 155–164

Quality or Quantity?

Quality or quantity? Is that the question?
Whether 'tis nobler in the mind,
To sling arrows at quantity for the sake of quality,
Or to take arms against quality to allow quantity.
Perhaps by opposing either, we end both.
To die, – to sleep, – no more.
And by a sleep to say we end both,
We may allow a consummation, devoutly to be wished,
To sleep, perchance to dream:
Maybe that's the solution!
But alas, there's a rub.
For without achievement there's no satisfaction.
Of what use is quality with insufficient quantity?
And of what use is quantity without sufficient quality?
Do not quality and quantity both improve with effort?
What artist develops his art without extensive practice?
What scientist can contribute reliably without repetition?
What grant gets funded without enough quality publications?
Moreover, under many circumstances, quantity is the best quality!
For example, one can't feed the Russian population with caviar.

Who would bear the whips and scorns of hard work,
Or the spurns that patient merit of the potentially unworthy takes,
When he himself might his quietus make with a bare bodkin?
Who would fardels bear, to grunt and sweat under a weary life,
But that benefits from quantity of quality should result?
Which professional, yea, did yet produce,
Without the sweat and blood of labour?
What inspiration was e'er expressed,
Without the discipline of learning?
Among the great human creators, do not quality and quantity coincide?
(Examine works of Bach, Beethoven and Brahms,
Or of Darwin, Einstein and Pauling, for example.)
And thus, it seems, the question is not quality or quantity,
But both or neither.
Let this perplexing fact not puzzle the will,
Nor let fear of perseverance make cowards of us all.
Be not sicklied o'er with the pale cast of thought,
So as thus, to loose the name of action.

Will Shakespeare
Milt Saier