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SERUM RESPONSIVENESS OF CARBOHYDRATE BINDING PROTEIN 35 EXPRESSION: COMPARISON BETWEEN HUMAN FIBROBLASTS OF DIFFERENT REPLICATIVE CAPACITIES

by

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ABSTRACT

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BY

Kimberly K. Hamann

We have compared the expression and localization of Carbohydrate Binding Protein 35 (CBP35) in human SL66 fibroblasts of different replicative capacities. When quiescent young (passage 11) SL66 cells were treated with serum, there was a dramatic stimulation in the expression of CBP35. This response was revealed both by an increase in the percentage of cells positively stained with anti-CBP35 under immunofluorescence, as well as by an increase in the amount of the protein in immunoblots. The rise in the expression of CBP35 in proliferating cells is manifested most clearly in the nuclear fraction, with elevation in the levels of the nonphosphorylated (pI 8.7) protein, as well as the phosphorylated (pI 8.2) derivative. In contrast, older (passage 31-35) cultures of SL66 fibroblasts appear to have lost the proliferation-dependent regulation of CBP35 expression. There was little or no down regulation of CBP35 protein upon serum deprivation of the high passage cells. Moreover, these cells also appeared to be unresponsive to serum stimulation, in contrast to that found in young, normal human fibroblasts. The nuclear, unphosphorylated form of the lectin was not found in any cultures of high passage SL66 cells.

To the Lord for the strength and knowledge he has provided

To my husband, David, for his love, support and patience

And to my family for their encouragement

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Abbreviations

CBP35	Carbhydrate Binding Protein 35
CLL	Chicken Lactose Lectin
CRD	Carbohydrate Recognition Domain
DME	Dolbecco modified Eagle's medium
EDTA	Disodium Ethylenediamine Tetraacetate
ER	Endoplasmic Reticulum
hnRNP	heterogeneous nuclear ribonucleoprotein complex
IEF	isoelectrice focusing
LDH	Lactate dehydrogenase
MEM	Eagle's minimal essential medium
NADH	Nicotinamide Adenine Dinucleotide reduced form
NEPHGE	nonequilibrium pH gradient electrophoresis
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
SDS	sodium dodecyl sulfate
SRE	Serum Response Element
SRF	Serum Response Factor
Tris	Tris (hydroxymethyl) aminomethane
WS	Werners Syndrome
XL	*Xenopus laevis* skin lectin

CHAPTER 1:
LITERATURE REVIEW

INTRODUCTION

The objective of this project was to compare the expression of Carbohydrate Binding Protein 35 (CBP; M_r 35,00) in cells with different replicative capacities. In order to address this question analysis was carried out: (a) at the level of single cells by immunofluorescence; (b) at the level of total cellular extracts by Western blotting; and (c) at the level of isoelectric variants by two-dimensional gel electrophoresis. The following chapter provides the background for this project.

Lectins: Definition and Classifications

Lectins were originally isolated from plant and animal sources on the basis of their saccharide binding (1,2,3). The present review focuses on animal lectins. The portion of the protein involved in the sugar binding has been defined as the carbohydrate recognition domain (CRD)(2). Some lectins also contain another domain, with properties not necessarily related to saccharide-binding (e.g. membrane anchorage). It has been hypothesized that many of these bifunctional lectins arose by the fusion of two separate genes.

There are mainly two categories of animal lectins, the C-type and the S-type (2). The C-type , or Ca^{2+} dependent, lectin is a protein which requires the presence of Ca^{2+} ions for carbohydrate binding activity. In addition, the pH of the solution must be above 6.5 and the disulfide bridges must be formed between the designated cysteines (shown in figure 1) for the CRD to be active. Thus far, all the C-type lectins are extracellular. The CRD is generally located on the carboxyl end of the molecule and there appears to be a conserved motif of 18 amino acid residues that mediates carbohydrate binding. Several of the C-type lectins are found in oligomeric structures, suggesting that they are multivalent (3).

There are several examples of the C-type lectin. A summary is found in figure 1. The hepatic lectins are found in a variety of species. Theyare mostly studied in the rat and chicken. The rat hepatic lectin is also known as asialoglycoprotein receptor because

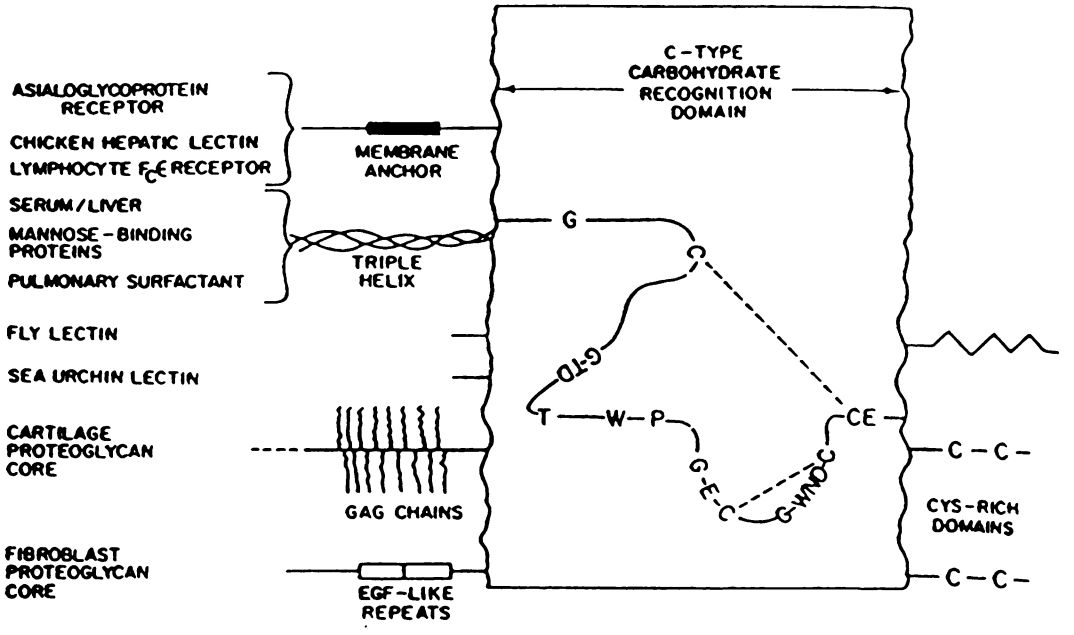


Figure 1 Summary of structural features of C-type animal lectins. The (nearly) invariant residues found in the commaon carbohydrate-recagnition domain of the C-type lectins are shown, flanked by schematic diagrams of the special effector domains (if any) found in individual members of the family (Referecne 2).

of its ability to remove desialylated glycoproteins from the circulatory system (4,5). The rat hepatic lectin CRD has specificity for N-acetyl galactosamine where as the chicken hepatic lectin CRD recognizes N-acetyl glucosamine (5,6). The amino terminal portion of the polypeptide is exposed to the cytosol. The carboxyl terminal portion contains the CRD and is exposed at the cell surface. The polypeptide thus contains a membrane spanning domain that goes through the plasma membrane (transmembrane domain)(7). The manose-binding protein is another C-type lectin which has specificity for manose (8). The amino terminus is collagen like because of a triplet motif Gly-X-Y; X is often proline and Y is frequently hydroxyproline (9,10). Yet other C-type lectins are proteoglycan cores. The cartilage proteoglycan core has many glycosaminoglycan side chains attached to its core at the amino terminal portion. It also contains a domain capable of binding hyaluronic acids that are associated with other proteins. (11,12). The fibroblast proteoglycan core has an amino terminus with an epidermal growth factor like-repeat; this may suggest capability for intercellular signaling (13). Thus, the C-type lectins consist of a wide range of proteins, with variations both in the amino terminal "effector" domain as well as in the carbohydrate-binding specificity of the carboxyl terminal CRD.

The S-type (soluble or thiol dependent) lectins do not have a divalent cation dependence in binding sugars. Rather their sulfhydral groups must be reduced in order to bind to saccharides. These lectins contain a CRD which consists of 39 highly conserved amino acid residues. The CRD of the S-type lectins usually have specificity for galactoside sugars. Many of these lectins are capable of forming dimers (3).

The S-type lectin family is further subdivided into two groups: (a) carbohydrate binding protein 13.5 (CBP13.5) and CBP16 fall into the 12-16 kDa subgroup and (b) the

other subgroup which consists of proteins in the 30-35 kDa range, of which CBP35 is member. Both of these subgroups are shown in figure 2. The 12-16 kDa group contains a single domain, the CRD. The 30-35 kDa group, on the other hand, contains a CRD in the carboxyl terminal portion and an "effector" domain in the amino terminal portion.

CBP35 has an amino terminal domain that is glycine and proline rich and shows some structure similarities to proteins of the heterogeneous nuclear ribonucleoprotein complex (hnRNP). The carboxyl terminal domain of CBP35 is homologous to the CRD of other galactoside binding S-type lectins (14). Other proteins from a variety of tissues/cell types appear to be identical or homologous to CBP35 in terms of its known structure: (a) L-34, isolated from murine fibrosarcomas as a metastasis-associated lectin (15); (b) Mac-2, identified as a cell surface marker for macrophage differentiation (16,17); (c) εBP, or IgE binding protein, isolated from rat basophilic leukemia cells (18); and (d) Laminin-Binding protein (LBP), the major non-integrin LBP from murine macrophage (19).

Localization

The subcellular distribution of lectins may vary. Some lectins have been shown to alter their localization depending on changes within the cell; three examples will be given where the lectin is later found outside the cell after a cellular response. Chicken lactose lectin I (CCL-I), a 13.5 kDa S-type lectin, has been shown to change its distribution dependent on the stage of differentiation in embryonic chick muscle cells (25,26). Through immunohistochemistry, CCL-I is located intracellularly in 8 day embryo

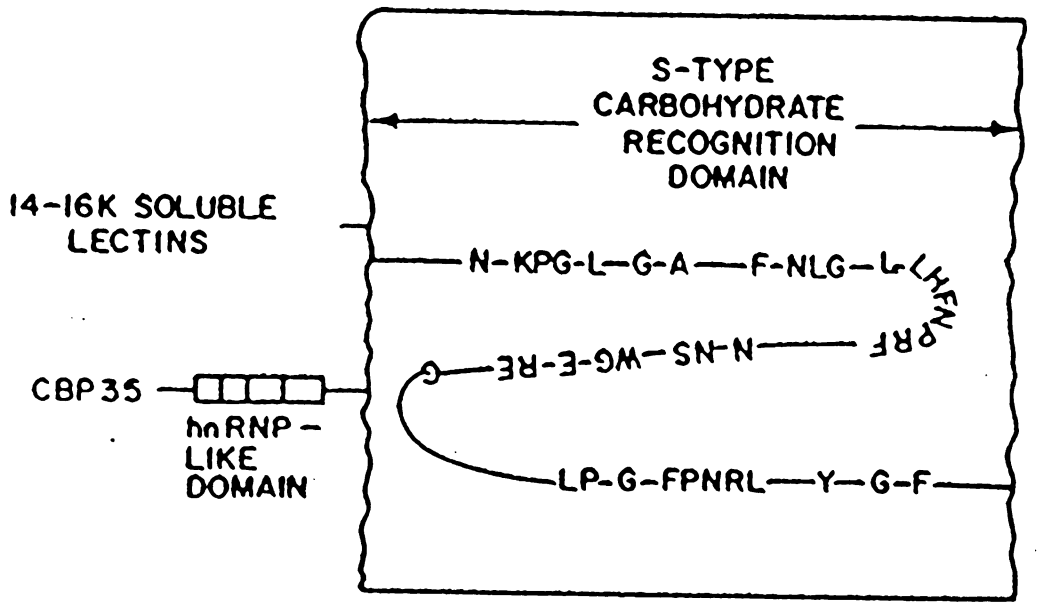


Figure 2: Summary of structural features of S-type animal lectins. Conserved residues found in all of the family members so far sequenced are shown. In addition, the extra domain found in CBP35 is shown schematically (Reference 2).

myoblasts but by the 12th day CCL-I is mostly extracellular upon formation of myotubes from cell fusion. In adult chickens, CCL-I is expressed at very low levels in pectoral muscle tissue (27). In contrast, embryonic liver tissue contains low amounts of CCL-I and as the chick matures to an adult the expression of CCL-I is found to increase in the lining of hepatic sinusoids (28). CLL-I's extracellular role is suggested to be involved in cell-cell or cell-matrix interactions by associating with proteoglycans. Chicken lactose lectin II (CCL-II), a 16 kDa S-type lectin, also is differentially localized during differentiation. CCL-II is found in secretory vesicles of goblet cells and is secreted with mucin when lactose or a cholinergic stimulant is present at low concentrations (28,30,31). Another lectin, *Xenopus laevis* skin lectin (XL), demonstrates a change in distribution due to an external stimulant. XL is a soluble cytoplasmic lectin (16kDa) but following an injection of epinephrine, XL is secreted along with many other small proteins (32). These three lectins have shown a change in distribution; this may suggest alternative roles for the same lectin.

Many extracellular lectins have been identified and may prove to have important roles. The Cartilage Proteoglycan core is found in the extracellular matrix of cartilage and it is believed to be involved in forming supramolecular aggregates via the hyaluronic acid binding site (33). The fibroblast chondoitin sulfate proteoglycan core is also distributed in the extracellular matrix and may participate in cell-cell or cell-matrix signaling because of the epidermal growth factor like repeat motif present in its amino acid sequence (34).

Other localization studies have shown that lectins are distributed within the cytoplasm. Two such lectins are calf lectin (12 kDa) and the chicken lectin (13 kDa) which were

found to be uniformily distributed in the cytoplasm (44). Previously mentioned, XL, a *Xenopus laevis* skin lectin, is normally found in the cytosolic compartment (32).

Subcellular distribution of lectins in the nucleus has also been suggested. Studies involving the use of fluorescently labeled neoglycoproteins in baby hamster kidney cells have shown that there are specific sugar binding sites found in the nuclei. Fluorescence analysis suggested that the sugar binding sites could be associated with RNP elements of the nuclei (45). CBP35 is found in the nuclei and has also been found to associate with heterogeneous nuclear ribonucleoprotein complex (hnRNP) proteins. Details of CBP35 will be covered in the following section.

Carbohydrate Binding Protein 35

CBP35 is a galactose specific lectin which was initially found in cultured 3T3 fibroblasts (50). It has since been found in a variety of mouse and human tissues, including human SL66 skin fibroblasts (51,52).

In confluent monolayer of 3T3 cells, CBP35 has been found on the cell surface (<5%), cytoplasm (90%) and nucleus (5%). Fluorescent studies, on the localization of CBP35, have shown that a distinct subset of 3T3 cells exhibit punctate nuclear staining (56). This lectin has been suggested to associate with hnRNP proteins. On cesium sulfate gradients, CBP35 was detected at a density corresponding to that reported for hnRNP. Nuclei from 3T3 cells treated with Triton X-100 release CBP35 when digested with ribonuclease A but not deoxyribonuclease I (54). Futhermore, the examination of the deduced amino acid sequence of a CBP35 cDNA clone has revealed a glycine- and

proline-rich amino terminal portion with structural similarites to regions of hnRNP proteins (14).

In other laboratories, some proteins have demonstrated a close if not identical structural relationship to CBP35. All these proteins have been seen on the cell surface: L34, Mac-2, LBP, εBP (16,17,18,19). The quantity of CBP35 seen on the surface of 3T3 fibroblasts is usually very low (<5% of total cellular CBP35). This introduces a most puzzling question. How does CBP35 get to the cell surface since no signal sequence has been detected for the exocytotic pathway? Several hypothesis have been presented to explain this dual localization: (a) if this is due to secretion there must be an alternative secretory pathway since there is no signal sequence directing it to the secretory pathway through the ER and golgi; otherwise, (b) there must be an alternative 5' splice site or start site that contains the signal sequence for the exocytotic pathway, or (c) because of the low levels in most cases, extracellular CBP35 cell distribution is due to ruptured cells. Other proteins with a defined extracellular role have been shown to also lack the signal sequence for secretion : (a) Basic Fibroblast Growth Factor, found in many bovine tissues has mitogenic effect on mesoderm derived cells, (b) Factor XIII located intracellularly in megakaryocytes and extracellularly is a blood coagulation protein, and (c) platelet derived endothelial cell growth factor is a mitogen for endothelial cells (46,47,48). This suggests that an alternative secretory pathway is highly probable.

Proliferation Dependent Expression of CBP35

The expression of CBP35 has been shown to be proliferation dependent in 3T3

fibroblasts. In quiescent cell, there is low level of CBP35 expressed and is mainly found in the cytoplasm. Where in proliferating cells there is an increase in the expression of CBP35 and it is predominantly in nucleus (53,56). Using a time course study, a significant increase in the level of CBP35 was identified between 4 and 8 hours after serum stimulation of serum-deprived cells (56). At the protein level, this lectin appears to be induced early in G1 phase.

Other time course studies have confirmed the proliferation dependent expression of CBP35 at the mRNA transcription level (55). Northern blots demonstrated a noticeable increase in the accumulation of mRNA within 30 minutes after serum addition, followed by a dramatic increase thirty minutes later. These data are supported by results from nuclear run off transcription assays. Thus it appears that the gene for CBP35 is turned on very early, shortly after the induction of c-fos (49).

Moreover, when serum stimulation was carried out in the presence or absence of cyclohexamide, the rate of transcription and accumulation of mRNA was comparable. In fact, an increase in the amount of detected mRNA was seen when cyclohexamide was present (55). Such results indicate that no synthesis of other gene products is required to stimulate transcription of CBP35. Thus, induction of CBP35 appears to be a primary event, presumably resulting from the signal transduction of growth factors.

The sequence of a clone for the CBP35 gene has shown that this gene contains a sequence (CCAATTAAGG) at the 5' flanking region that fits the consensus (CC $(A/T)_6$ GG) termed the serum response element (SRE) (57). SRE was initially found in c-fos and is now found in many β-actin genes. It has also been demonstrated that c-fos is regulated by serum (58). In addition, there is an upstream site containing the SRE

consensus sequence in the c-fos gene (-313 to -306) that has been shown to induce transcription upon serum stimulation (59,60). CBP35 may be regulated by an SRE and/or it may be regulated by another means. Whichever the case, CBP35 expression has demonstated to be proliferation dependent. This is the focus of my research.

In Vitro Senescence of Cultured Human Cells

Normal human cells have a finite replicative capacity in vitro (61,62). As cells continue to divide they will eventually enter a stage where they no longer proliferate. This is the state of senescence; it is irreversible (63). The in vitro senescence of cultured human cells has been used as a model for the study of the aging process in cells, mainly on the basis of the following correlations. First, the number of passages (population doublings) is proportional to the life span of the donor (65). For example, a mouse has a much shorter life span then a human being, so the proliferation capacity (number of population doublings) of mouse cells in vitro would be significantly less than human cultures. Second, the proliferative capacity of cultures is inversely proportional to the age of the donor cells (64). An example of this is infant human tissues in culture have more proliferative capacity then adult tissues in culture. Lastly, the average number of population doubling for normal human cells is fifty (61,62).

It has been of great interest to understand what causes senescence. Some believe that there is a molecule in senescent cells that inhibits replication by interfering with a particular process. Others think there is a deficiency in senescent cells so that replication doesn't occur because of the absence of an essential molecule.

Evidence that anti-proliferating agents are present in senescent cells are shown in several studies. Cell fusion studies best demonstrate this. Fusion of young proliferating fibroblasts to senescent fibroblasts registered a decrease in DNA synthesis of the young proliferating cells, measured by tritiated thymidine incorporation (66,67). Similar results were seen with immortalized cells, HeLa cells (68). When poly (A)+ RNA from senescent cells were injected into young proliferating cells, DNA synthesis was inhibited (69). These results suggest that there is one or more proteins expressed in senescent cells that prevent DNA synthesis.

In senescent cells, there are some studies that demonstrate an absence in certain enzymatic activities or particular proteins. An example of a mitogenic response in proliferating cells is the association of epidermal growth factor (EGF) associates with the EGF-receptor (EGF-R), which results in autophosphorylation of the EGF-R in response to EGF binding (34,36). This kinase activity is not detected in senescent WI38 cells; however, the autophosphorylation of the platelet derived growth factor-receptor (PDGF-R) does occur when PDGF interacts with PDGF-R (70,71). It also has been shown that PDGF induces c-fos and c-myc expression in proliferating cells (29,58). Senescent cells do not express c-fos when serum stimulated, but c-myc expression is similar to that of proliferating cells (24). Normally c-fos is induced fifteen to thirty minutes after serum addition; it is one of the first genes to be turned on after stimulation (21,22,23). The lack of c-fos activation in senescent cells may be the reason that the cells are in a nonreplicative state since this may eliminate a cascade of events. Several other proteins that are expressed in proliferating cultures are not seen in senescent cultures: (a) replication dependent histones, and (b) nuclear antigen K-67 (24,71). These proteins are

expressed during S phase, so after c-fos which is expressed in G_1. Other S phase proteins have been expressed in senescent cells, DNA polymerase alpha and thymidine kinase (72,73). Many differences have been identified between replicating cultures and senescent cultures. The cause of senescence may in part be due to the repression of c-fos which in turn may be repressed by an anti-proliferating agent.

Premature aging cultures have also been studied. Werners Syndrome (WS) is an autosomal recessive human genetic disorder which is characterized by severe premature aging signs shown in Table 1. The WS and aging don't represent the same process; although, they share many of the same features (78). Studies have been done in comparing WS to normal human diploid cells. The WS patients have a more limited life span (40). Therefore, the WS cells have a lower proliferative capacity in culture then the normal human cells (74). Through a cell cycle study, it was revealed that the WS cells have a cell cycle which is longer then the average growth rate for human cultures. The extended length in the growth rate was observed to be due to a prolonged S phase. DNA alpha polymerase was compared in WS and normal human fibroblasts. The polymerase had the same rate of elongation in both cultures; however, the initiation rate was less in WS cells (76).

It has been shown that WS cells undergo a very similar but accelerated senescence compared to normal human cells (39,40). Fusion studies were done on WS cells of low and high passage along with senescent normal human fibroblast cultures. The WS cells were fused to normal human cells and HeLa cells. A decrease in the normal human cells and HeLa cell DNA synthesis was seen in all fusions to WS cells, but the most significant

Table 1. Similar characteristics of Werner's Syndrome and aging (R. 37)

Very similar:

1. Atherosclosis, artiosclerosis and medical calcinosis
2. Grayin of the hair
3. Hypermelanosis
4. cerebral cortical atrophy
5. Lymphoid depletion and thymic atrophy

More severe in Werner's Syndrome

1. Calcification of valve rings and leaflets
2. Hyalinization of seminiferous tubes
3. Atrophy of skin appendages
4. Osteoporosis of distal extremities

decreases were in cell fusions to high passage and senescent WS cells(43). These results are similar to the fusion studies on senescent normal human cells. The morphology of senescence appears to be the same, irregular shape and accumulated cytoplasmic fibrils (33).

In conclusion, the significance of utilizing human tissues for studing aging has been demonstrated by looking at senescence in normal human tissues as well as premature aging cultures (e.g. WS). This has been shown through fusion studies, enzyme studies, and expression studies.

1. Goldstein, I. J., Hughes, C., Monsigny, A., Osawa, T., Sharon, N. 1980. Nature(London) 285: 66-68.

2. Drickamer, K. 1988. J. Biol. Chem. 363: 9557-9560.

3. Barondes, S. A. 1988 Trends Biochem. Sci. 13: 480-492.

4. Ashwell, G., Morell, A. G.1974. Adv. Enzymol. 41: 99.

5. Ashwell, G., Harford, J. 1986. Annu. Rev. Biochem. 51: 531-554.

6. Halberg, D. F., Wagner, R. E., Farrell, D. C., Hildreth, J., Quesenberry, M. S., Drickamer, K. 1987. J. Biol. Chem. 262: 9828-9838.

7. Chiacchia, K. B., Drickamer, K. 1984. J. Biol. Chem. 259: 15440-15446.

8. Wild, J., Robinson, D., Winchester, B. 1983. Biochem. J. 210: 167-174.

9. Taylor, M. E., Summerfeld, J. A. 1986. Clin. Sci. (London). 70: 534-546.

10. Drickamer, K., Derdeil, M. S., Reynolds, L. 1986. J. Biol. Chem. 261: 6878-6887.

11. Doege, K., Ferandez, P., Hassell, J. R., Sasaki, M., Yamada, Y. 1986. J. Biol. Chem. 261: 8108-8111.

12. Halberg, D. F., Prouex, G., Doege, K., Yamada, Y., Drickamer, K. 1988. J. Biol. Chem. 263: 9486-9490.

13. Krusuis, T., Gehlsen, K. R., Ruoslahti, E. 1987. J. Biol. Chem. 262: 13120-13125.

14. Jia, S., Wang, J. L. 1988. J. Biol. Chem. 263: 6009-6011.

15. Raz, A., Lotan, R. 1981. Cancer Res. 263: 3642-3647.

16. Ho, M. K., Springer, T. A. 1982. J. Immunol. 128: 1221-1228.

17. Cherayil, B. J., Weiner, S. J., Pellai, S. 1989. J. Exp. Med. 170: 1959-1972.

18. Albrandt, K., Orida, N. K., Lui, F. T. 1987. Proc. Natl. Acad. Sci. USA. 84: 6859-6862.

19. Woo, H.-J.,Shaw, L. M., Messier, J. M., Mercurio, A. M. 1990. J Biol. Chem. 265: 7097-7099.

20. Gabius, H.-J., Nagel, G. A. 1988. in Lectins and Glycoconjugates in Oncology. H.-J. Gabius, G. A. Nagel. ed. Springer-Verlay. New York. pp. 244.

21. Holt, J. T., Venkat Gopal, T., Mou Hon, A. D., Nienhuis, A. W. 1986. Proc. Natl. Acad Sci. USA. 83: 479-482.

22. Nishikura, K., Murray, J. M. 1987. Mol. Cell Biol. 7: 639-647.

23. Riabowol, K. T., Vosatk, R. J., Ziff, E. B., Lamb, N. J., Feramisco, J. 1988. Mol. Cell Biol. 8: 167-173.

24. Seshadri, T., Campisi, J. 1990. Science. 247: 205-209.

25. Sharon, N., Liz, H. 1989. Science. 246: 227-234.

26. Teichberg, V., Silman, I., Beitsch, D. D., Resheff, G. 1975. Proc. Natl. Acad. Sci. USA. 72: 1383-1387.

27. Barondes, S. H. 1986. in The Lectins: Properties, Functions and Aplications. I. E. Liener, N. Sharon, I. J. Goldstein. ed. Academic Press Orlando, FL. pp. 437-466.

28. Beyer, E. C., Tokuyasu, K. T., Barondes, S. H. 1979. J. Cell Biol. 82: 565-571.

29. Paulsson, Y., Bywater, M., Heldin, C.-H., Westermark, B. 1987. Exp. Cell Res. 171:186-194.

30. Bols, N. C., Roberson, M. M., Haywood-Reid, P. L., Cerra, R. F., Barondes, S. H. 1986. J. Cell Biol. 102: 492-499.

31. Beyer, E. C., Barondes, S. H. 1982. J. Cell Biol. 97: 1875-1881.

32. Robinson, M. M., Barondes, S. H. 1983. J. Cell Biol. 97: 1875-1881.

33. Salk, D., Bryant, E., Hoehn, H., Johnston, P., Martin, G. M. 1985. in Werners Syndrom and Human Aging. D. Salk, Y. Fujiwara, G. Martin. ed. Plumen Press, New York. pp. 305-311.

34. Berridge, M. J. 1985. Sci. Am. 253: 142-152.

35. Bowman, T. E., Balain, G. 1989. Matrix Res. 9: 99-108.

36. Schlessinger, J., Lax, I., Gill, S., Kris, R., Libermann, T. A., Bar-Eli, M., Harari, I., Yarden Y. in Mechanisms of Receptor Regulation. by G. Poste, S. T. Crooke. ed. Plenum Press, New York. pp. 37-51.

37. Epstein, C. J., Martin, G. M., Schultz, A. L., Motulsky, A. G. in Werners Syndrome and Hauman Aging. by D. Salk, Y. Fujiwara, G. M. Martin ed. Plenum Press, New York. pp. 57-120.

39. Goldstein, S. 1979. J. Invest. Dermatol. 73: 19-23.

40. Salk, D., Bryant, E., Au, K., Hoehn, H., Martin, G. M. 1981. Hum. Genet. 58: 310-316.

41. Levi, G., Teichberg, V. I. 1983. Immuno. Lett. 7: 35-39.

42. Sharon, N. 1983. Adv. Immunol. 34: 213-215.

43. Rabinovitch, P. S., Norwood, T. H. 1980. Exp. Cell Res. 130: 101-109.

44. Briles, E. B., Greogory, W., Fletcher, P., Kornfelds, S. 1979. J. Cell. Biol. 81: 528-537.

45. Seve, A. P., Hubert, J., Bouvier, D., Bougeiois, C., Midouxs, P., Roche, A. C., Monsigny, M. 1986. Proc. Natl. Acad. Sci. USA. 83: 5997-6001.

46. Abraham, J. A., Mergia, A., Whang, J. L., Tunolo, A., Friedman, J., Hjerreld, K. H.,Gospodarowicz, D., Fiddes, J. C. 1986. Science. 233: 545-548.

47. Grundmann, U., Amann, E., Zittlmeissl, G., Kupper, H. A. 1986. Proc. Natl. Acad. Sci. USA. 83: 8024-8028.

48. Ishikawa, F., Miyozono, K., Hellman, U., Drexer, H., Wernstedt, C., Hagiwara, K.,Usai, K., Takaku, F., Risau, W., Heldin, C.-H. 1989. Nature. 338: 557-561.

49. Muller, R., Bravo, R., Burckhandt, J., Curran, T. 1984. Nature. 312: 716-721.

50. Roff, C. F., Wang, J. L. 1983. J. Biol. Chem. 258: 10657-10663.

51. Roff, C. F., Rosevear, P. R., Wang, J. L., Barker, R. 1983. Biochem J. 211: 625-629.

52. Crittenden, S. L., Roff, C. F., Wang, J. L. 1984. Mol. Cell Biol. 4: 1252-1259.

53. Moutsatsos, I. K., Davis, J. M., Wang, J. L. 1986. J. Cell Biol. 102: 477-483.

54. Laing, J. G., Wang, J. L. 1988. Biochem. 27: 5329-5334.

55. Agrwal, N., Wang, J. L., Voss, P. G. 1989. J. Biol. Chem. 264: 17236-17242.

56. Moutsatsos, I. K., Wade, M., Schindler, M., Wang, J. L. 1987. Proc. Natl. Acad. Sci. USA. 84:6452-6456.

57. Gustafsen, T. A., Boxer, L. M., Kedes, L. 1988. Mol. Cell Biol. 8: 4110-4119.

58. Greenberg, M. E., Ziff, E. B. 1984. Nature (London). 311: 433-438.

59. Treisman, R. 1986. Cell. 46: 567-574.

60. Treisman, R. 1985. Cell. 42: 889-902.

61. Hayflick, L., Moorhead, P. A. 1961. Exp. Cell Res. 25: 585-621.

62. Hayflick, L. 1965. Exp. Cell Res. 37: 614-636.

63. Stanulis-Praeger, B. M. 1987. Mech. Aging Dev. 38: 1-48.

64. Schneider, E. L., Mitsui, Y. 1976. Proc. Natl. Acad. Sci. USA. 73: 3584-3588.

65. Rome, D. 1981. Proc. Natl. Acad. Sci. USA. 78: 5009-5013.

66. Norwood, T. H., Pendergrass, W. R., Sprgue, C. A., Martin, G. M. 1974. Proc. Natl. Acad. Sci. USA. 71: 2231-2235.

67. Burmer, G. L., Norwood, Z., Norwood, T. 1982. J. Cell Biol. 94: 187-192.

68. Rabinovich, P. S., Norwood, T. H. 1980. Exp. Cell Res. 130: 101-109.

69. Lumpkin, C. K., McClung, J. K., Pereira-Smith, O. M., Smith, J. R. 1986. Science. 232: 393-395.

70. Carlin, L. R., Phillips, P. D., Knowles, B. B., Cristofalo, V. J. 1983. Nature. 306: 617-620.

71. Paulsson, Y., Bywater, M., Pfeifer-Ohlssen, S., Ohlssen, R., Nilsson, S., Heldin, H., Westermark, B., Betsholtz, C. 1986. EMBO J. 5: 2157-2162.

72. Olashaw, N. E. 1983. Exp. Cell Res. 149: 547-554.

73. Pendergrass, W. R., Saulewicz, A. C., Salk, D., Norwood, T. 1985. J. Cell Phys. 124: 331-336

74. Thompsen, K. V. A., Holiday, R. 1983. Genontology. 29: 73-82.

75. Mitsui, Y., Yamamoto, K., Yamamoto, M., Matuoka, K. 1985. in Werners Syndrome and Human Aging. D. Salk, Y. Fujiwara, G. Martin. ed.Plenum Press, New York, NY. pp. 567-586.

76. Funeo, H., Yamada, M., Takeuchi, F., Goto,M., Miyamato, T., Hori, T. 1985. in Werners Syndrome and Human Aging. D. Salk, Y. Fujiwara, G. Martin. ed. Plenum Press, New York, NY. pp. 341-351.

77. Keyoji, T., Yamamura, K., Fukuchi, K., Kawai, K., Kumahara,Y. 1985. in Werners Syndrome and Human Aging. D. Salk, Y. Fujiwara, G. Martin. ed. Plenum Press, New York, NY. pp. 341-351.

78. Martin, G. M. 1982. Syndroms of accelerated aging, Nat. Cancer Instit. Monog. No. 60:241-247.

Chapter 2:

Expression of Carbohydrate Binding Protein 35 in Human Fibroblasts: Senescent Cultures Lack the Nonphosphorylated Nuclear Form*

INTRODUCTION

Carbohydrate Binding Protein 35 (CBP35; M_r 35,000) is a galactose-specific lectin found in many cells and tissues (1,2). The amino acid sequence, deduced from the nucleotide sequence of a cDNA clone (3), showed that the polypeptide most likely consists of two domains. The COOH-terminal half was homologous to many other β-galactoside-specific lectins; in particular, the conserved amino acid residues indicated that CBP35 belonged to the S-type family of lectins (4). The NH_2-terminal half was proline- and glycine-rich and exhibited some structural similarity to proteins of the heterogeneous nuclear ribonucleoprotein complex (hnRNP). Indeed, CBP35 has been identified in several subcellular locations of mouse 3T3 fibroblasts, predominantly in the cytoplasm and the nucleus (5). Analysis of the nucleoplasm by density gradient sedimentation and by immunoblotting suggested that CBP35 is a component associated with the ribonucleoprotein element of the cell, possibly the hnRNP complex (6).

When quiescent 3T3 cells were stimulated by the addition of serum, there was an increased expression of CBP35 as monitored by: (a) analysis at the single cell level by immunofluorescence (7); (b) analysis at the protein isoelectric species level by subcellular fractionation, two-dimensional gel electrophoresis, and immunoblotting (8); (c) analysis at the mRNA level by Northern blotting (9,10); and (d) analysis of the transcription of the CBP35 gene in nuclear run-off assays (10). We have recently determined the structure of the murine CBP35 gene (11). This structure revealed a 10-nucleotide

sequence, located in the 5′ flanking region, that is consistent with the consensus sequence for the regulatory structure designated serum response element (SRE) (12,13). Thus, this SRE-like sequence may serve as a binding site for specific transcription factors such as the serum response factor (SRF) and may account for the above observations on serum-stimulated expression of CBP35.

CBP35 is also found in human fibroblasts (14,15). Because these human fibroblasts have a finite life-span in culture, there is a well-documented, age-acquired difference in replicative capacity between early passage (young) and late passage (old) cells (16,17). This offered a unique opportunity to compare the expression of CBP35 in cells with different responsiveness, in terms of DNA replication, to serum stimulation. In preliminary studies (15), we had found that the expression of CBP35 in late passage human SL66 fibroblasts was unresponsive to serum stimulation, in contrast to that found in early passage normal human fibroblasts and in 3T3 cells. We now document these observations on a quantitative basis. In addition, we also delineate one key aspect of the difference between CBP35 in young versus old cells: the absence of the unphosphorylated CBP35 protein in the nucleus of the old cells.

EXPERIMENTAL PROCEDURES

Cell Culture

Swiss 3T3 fibroblasts (CCL92, American Type Culture Collection) were cultured in Dulbecco modified Eagle's medium (Gibco) containing 10% (v/v) calf serum as described previously (10). Normal human fibroblasts, SL66 (18) and KD, were gifts from Drs. J. J. McCormick and V. M. Maher (Michigan State University). Werner's Syndrome (WS) fibroblasts AG6300 were purchased from NIA Aging Cell Repository (Camden, NJ). The KD cells represent age-matched controls to the WS fibroblasts; the age of the KD donor was 35 years. The human cells were all cultured in Eagle's minimal essential media (Gibco) containing 20% (v/v) fetal calf serum (19).

Sparse cultures of these cells were seeded at a density of 1.0×10^4 cells/cm^2 in their respective culture medium. The cultures were synchronized by washing twice in media without serum and then incubated for 48 hours in their corresponding media containing 0.2% (v/v) serum. The 3T3 cultures were stimulated by the addition of calf serum to 10% (v/v) for 16 hours (7). The human cells were stimulated for 17 hours with 20% (v/v) fetal calf serum (15).

Quantitation of Immunofluorescent Cells

3T3 and human fibroblasts were seeded onto sterile coverslips as described above. Quiescent and proliferating cultures were washed with phosphate buffered saline (.14 M NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and fixed onto the coverslips with a 1 minute incubation in cold 70% acetone - 30% methanol. After

washing, the coverslips were blocked with 3% bovine serum albumin in Tris-buffered saline (20 mM Tris, pH 7.5, 0.5 M NaCl). They were then incubated in anti-CBP35 (1:50 dilution of polyclonal rabbit antiserum) or preimmune serum (1:50 dilution) for 1 hour at room temperature and washed, followed by incubation in fluorescein-conjugated goat anti-rabbit immunoglobulin (5,7).

For quantitation of percent fluorescently-labeled cells, an arbitrary threshold level was established to distinguish fluorescent cells from nonfluorescent cells. This threshold level was comparable to the staining obtained with preimmune serum. For all coverslips, several fields were counted until 100 total cells were found.

Preparation of Extracts and Subcellular Fractions

For whole cell extract preparation, the cells were washed in phosphate-buffered saline, scraped off the culture dishes, centrifuged (1330 x g, 3 minutes) and resuspended in 10 mM Tris (ph 7.5) containing 2 mM EDTA, 1 μg/ml soybean trypsin inhibitor, 1 U/ml aprotinin and 1 μg/ml leupeptin. The cells were sonicated three times for 15 seconds.

For the isolation of subcellular fractions, the cells were harvested in the same way, but resuspended in TKM buffer (20 mM Tris (pH 7.2), 5 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 μg soybean trypsin inhibitor, 1 U/ml aprotinin, 1 μg/ml leupeptin). The cells were lysed in a Dounce homogenizer and centrifuged (375 x g, 10 minutes). The pellet, which contained mostly nuclei with some unbroken cells, was aspirated through a 25-gauge needle and the resulting lysate was resedimented to obtain the nuclear pellet fraction. The two supernatants, which contained the post-nuclear fraction, were subjected

to a higher speed centrifugation (150,000 x g, SW50.1, 1 hour) to yield a supernatant (cytosolic fraction) and a precipitate (the membranous fraction) (8,20).

The total cell extracts, cytosolic fractions and nuclear fractions were measured for protein concentration by the Bradford assay (21). The purity of the subcellular fractions was assessed using enzyme assays for lactate dehydrogenase (LDH, EC 1.1.1.27) (22), a cytoplasmic marker, NADH-diaphorase (EC 1.6.99.1) (23), a marker enzyme for the endoplasmic reticulum, and the DNA binding assay for the dye, Hoechst 33258 (24). In addition, immunoblotting analysis was also carried out using a rabbit antiserum directed against pig muscle LDH (5); this antiserum was a gift from Dr. J. E. Wilson (Michigan State University).

One- and Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out by using isoelectric focusing (IEF) and nonequilibrium pH gradient (NEPHGE) electrophoresis in the first dimension, followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) in the second dimension (25,26). The IEF gels were prepared using 2.06 pH 3-10 ampholines (Pharmacia) and run for 10 min at 500 V and 3.5 hours at 750 V. In NEPHGE gels, the proportion of pH 3-10 ampholines to pH 7-9 ampholines was 2:1. The running conditions were as follows: the basic electrode solution was 50 mM NaOH; the acidic electrode solution was 25 mM H_3PO_4; the running times were 60 minutes at 400 V, 75 minutes at 650 V, and 15 minutes at 750 V using a mini-gel system (BioRad). The samples were dissolved in 9.5 M urea sample buffer and aliquots containing equal amounts of protein (~ 100 μg) were loaded per gel.

SDS-PAGE analysis was performed according to the Laemmli procedure (27). After electrophoresis, the proteins were transferred onto Immobilon-P (Millipore) (28). The blots were blocked overnight in Tris-buffered saline containing 3% (v/v) bovine serum albumin, incubated with anti-CBP35 (1:150 dilution) or with anti-LDH (1:150 dilution), washed in Tris-buffered saline containing 0.2% (v/v) Tween-80, and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (BioRad). The blots were quantitated using a Gelman densitometer, following methods described previously (7,8).

RESULTS

CBP35 Expression in Human Fibroblasts in Response to Serum Stimulation

When serum-starved cultures of mouse 3T3 fibroblasts were stimulated by the addition of serum, there was an increase in the level of CBP35, as detected by immunofluorescence staining of the cells 16 hours after serum addition. This was reflected both by an increase in the intensity of staining with rabbit anti-CBP35 antibodies (Figure 1), as well as by an increase in the percentage of cells exhibiting detectable fluorescence (Table I). Serum-starved, quiescent 3T3 cells showed little fluorescence staining; of the stained cells (~ 35%), some showed labeling of the nucleus (Figure 1). Sixteen hours after serum stimulation, 98% of the cells showed fluorescent staining, with the majority (~ 85%) of the fluorescent cells stained in the nucleus. Punctate intranuclear staining was prominent, as well as a distinct nuclear periphery.

We carried out, in parallel, the same experimental protocol on a human cell strain designated KD. The staining with anti-CBP35 was weak for serum-starved KD cells (Figure 1). Approximately half of the cells examined exhibited such weak fluorescence (Table I). Upon serum stimulation, the overall percentage of stained KD cells increased to 91%. The intensity of fluorescence in the stained cells also increased (Figure 1). Control experiments using preimmune serum were also carried out on the KD cells. There was negligible fluorescence due to preimmune serum in serum-starved, as well as in serum-stimulated KD cells (Figure 1). These results indicate that the expression of CBP35 in human KD fibroblasts was dependent on the proliferation state of the cell culture, as was observed in mouse 3T3 cells.

Figure 1: **Comparison of the immunofluorescence staining patterns of CBP35 in serum-starved, quiescent cultures (-) and cultures 16 or 17 hours following serum stimulation (+).** ph, phase contrast; fl, fluorescence; 3T3, mouse 3T3 fibroblasts; Werner, human fibroblasts of a Werner's Syndrome patient; KD, fibroblasts from a normal human age-matched with the Werner Syndrome patient. The last column represents the staining of human KD cells with preimmune rabbit serum in place of rabbit antiserum directed against CBP35. Immunofluorescence was carried out on methanol-acetone fixed cells using rabbit anti-CBP35 (1:50 dilution) or preimmune rabbit serum (1:50 dilution) and revealed with fluorescein-conjugated goat anti-rabbit immunoglobulin.

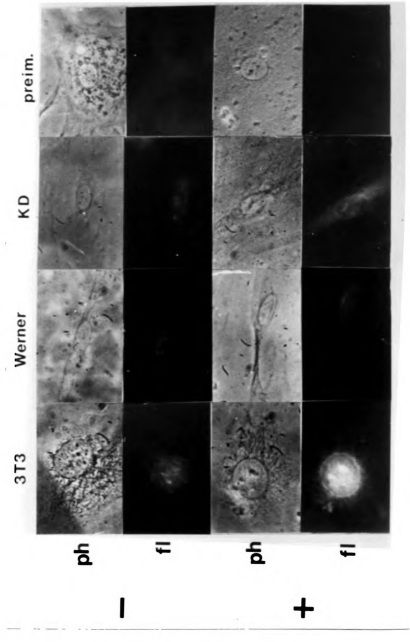

3T3
Werner
KD
preim.
ph
fl
ph
fl
-
+

Table I. Immunofluorescence comparison of CBP35 expression in mouse and human fibroblasts*

Cell Type	Serum-Starved (Q)	Serum-Stimulated (P)
3T3	35	98
WS	61	39
KD	56	91

* The slides, from which representative photographs were taken for Figure 1, were subjected to counting and the numbers represent the percent of fluorescently-labeled cells stained with anti-CBP35.

The behavior of human fibroblasts derived from a patient with Werner's Syndrome (WS) was somewhat different from that of KD cells, in terms of the expression of CBP35 as detected by immunofluorescence. There was weak staining in about 61% of the cells in serum-starved cultures (Figure 1 and Table I). Addition of serum, however, failed to increase the level of CBP35, neither in the intensity of staining in individual cells nor in the percentage of fluorescently labeled cells. In fact, quantitation of the latter parameter indicated that the percent of CBP35 positive cells in immunofluorescence decreased to 39% (Table I). Inasmuch as WS represents a heritable disease characterized by segmental premature aging (29), it was of interest to compare the levels of CBP35 in a strain of human fibroblasts that has been aged in culture, i.e. by serial passage of embryonic fibroblasts. The normal human KD fibroblasts were used in the above study because they represented an aged-matched control for the WS fibroblasts that were available. For the study of CBP35 expression in fibroblasts of different passage numbers, another normal human cell strain, SL66, was chosen because its history, in terms of number of *in vitro* doublings, was known.

Expression of CBP35 in SL66 Cells of Different Passage Number: Comparison by Immunofluorescence

The staining for CBP35 was weak for serum-starved SL66 cells at low, intermediate, and high passage numbers (passage 11, 17, and 31, respectively) (Figure 2). Using the weak fluorescence as a criteria, we found only 8% of the young (passage 11) cells stained with anti-CBP35. About half of the passage 17 and passage 31 cells examined were judged to be stained (Table II).

Figure 2: **Comparison of the immunofluorescence staining patterns of CBP35 in serum-starved, quiescent cultures (-) and cultures 17 hours following serum stimulation (+).** ph, phase contrast; fl, fluorescence. Human SL66 fibroblasts at passage number 11, 17, and 31 were analyzed. The procedure described in the legend to Figure 1 was used.

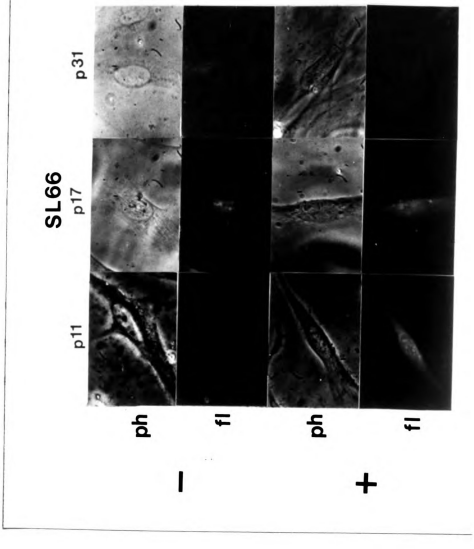

SL66
p11
p17
p31
ph
fl
ph
fl
-
+

Table II. Immunofluorescence comparison of CBP35 expression in SL66 cultures of different passages*

Passage Number	Serum-Starved (Q)	Serum-Stimulated (P)
P11	8	98
P17	50	96
P31	55	63

* The slides, from which representative photographs were taken for Figure 2, were subjected to counting and the numbers represent the percent of fluorescently-labeled cells stained with anti-CBP35.

Upon serum stimulation, the percent of fluorescent cells in passage 11 cultures increased from 8 to 98%. In individual cells, the overall staining intensity increased dramatically, with some exhibiting punctate intranuclear staining as well as a distinct nuclear periphery (Figure 2). Similar results were also obtained for passage 17 cultures of SL66 cells. There was an increase in staining intensity in individual cells and the percent labeled cells increased to 96% (Table II). These results, obtained with SL66 fibroblasts, are consistent with the corresponding observations made on normal human KD fibroblasts and mouse 3T3 cells.

Quiescent high passage SL66 cells (passage 31) have a moderate staining intensity and in some cases have no nuclear staining. Old SL66 cells yielded no significant increase in immunofluorescence of CBP35 upon serum stimulation, either in terms of staining intensity in individual cells or in terms of percent of labeled cells (Figure 2 and Table II). Thus, older SL66 cells (passage 31) mimicked the behavior of WS fibroblasts in the expression of CBP35 as a function of serum addition. Overall, the present results confirm our previous observations on CBP35 immunofluorescence in SL66 and WS fibroblasts (15). More importantly, however, we have provided a quantitative basis for comparison between cells of different passage numbers and between quiescent and proliferative populations.

Expression of CBP35 in SL66 Cells of Different Passage Number: Comparison by Immunoblotting

To compare the relative abundance of CBP35 in SL66 cells as a function of passage number and before and after serum stimulation, extracts of SL66 cells cultured

under the various conditions were subjected to SDS-PAGE and immunoblotting. A single polypeptide, migrating at a position corresponding to human CBP35 (14,15) and slightly faster than recombinant mouse CBP35, was observed in all the extracts (Figure 3). The intensity of the CBP35 band was quantitated by densitometric scanning and the results are summarized in Table III. Since equal amounts of total protein (~ 100 μg) from each extract were electrophoresed in individual lanes, the intensity of the immunoblotted band reflects the amount of CBP35 per unit of total cellular protein in each sample.

In serum-starved, quiescent cultures of SL66 cells, the amount of CBP35 (per mg of cellular protein) increased with increasing passage number of the culture. Thus, the specific activity of CBP35 in passage 32 cells was about 9 times higher than in passage 11 cells (Table III). This is reflected, in part, by a higher percentage of anti-CBP35 positive cells under immunofluorescence in the older SL66 cells (Table II). The addition of serum to the SL66 cultures markedly increased the amount of CBP35 in young (passage 11) cells; this increase was approximately 9-fold on a per mg total protein basis (Figure 3 and Table III). In the immunofluorescence analysis, both the intensity of anti-CBP35 staining in single cells, as well as the overall percentage of stained cells increased (Figure 2 and Table II). In intermediate passage cells (passage 17), there was a moderate increase in the amount of CBP35 upon serum stimulation. This most probably reflects the increase in the percent of fluorescently-labeled cells in serum-stimulated passage 17 cultures.

Finally, serum addition to passage 32 cultures of SL66 fibroblasts decreased the amount of CBP35 (Figure 3 and Table III). Since the percent of anti-CBP35 positive cells under immunofluorescence was apparently not altered by serum stimulation of the

Figure 3: **SDS-PAGE and immunoblotting analysis for CBP35 in extracts of human SL66 fibroblasts at passage 11 (lanes 1 and 2), passage 17 (lanes 3 and 4), and passage 32 (lanes 5 and 6).** The odd-numbered lanes contained extracts derived from serum-starved, quiescent cultures. The even-numbered lanes contained extracts derived from cultures 17 hours after serum addition. Each lane contained 10 μg of protein. Immunoblotting was carried out with rabbit anti-CBP35 (1:150 dilution) and revealed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. The position of migration of recombinant mouse CBP35 is shown as a reference.

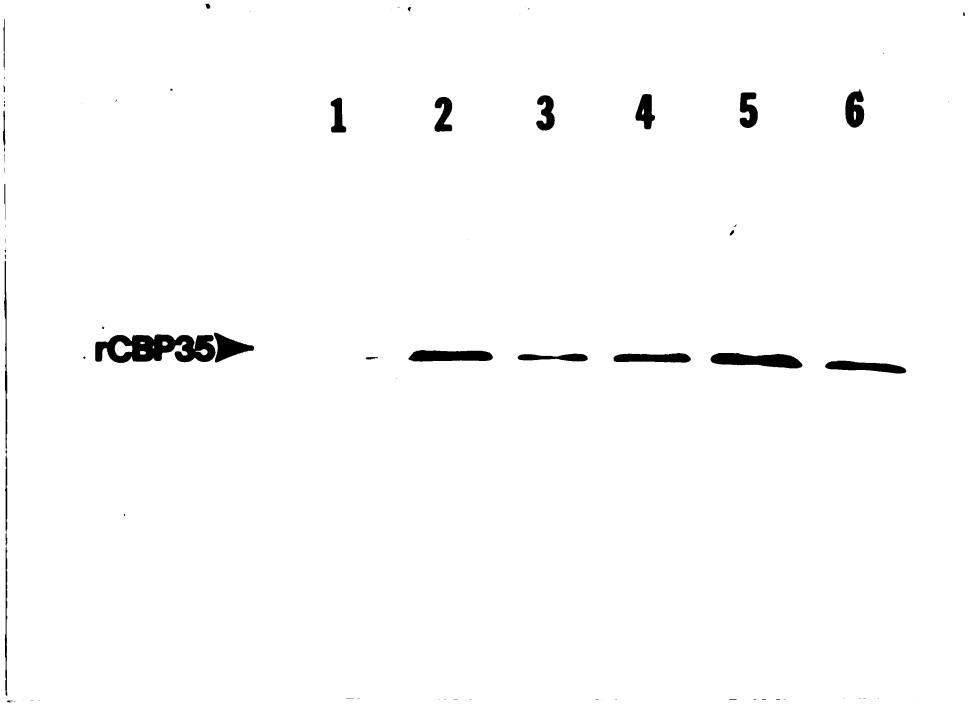

1
2
3
4
5
6
rCBP35

Table III. Quantitative comparison of the levels of CBP35 in SL66 cultures of different passages*

Extract	Serum-Starved (Q)	Serum-Stimulated (P)
P11	1.0	9.0
P17	4.3	6.8
P32	9.2	4.3

* The immunoblots shown in Figure 3 were subjected to densitometric scanning and the intensities of the bands are expressed as arbitrary units of CBP35.

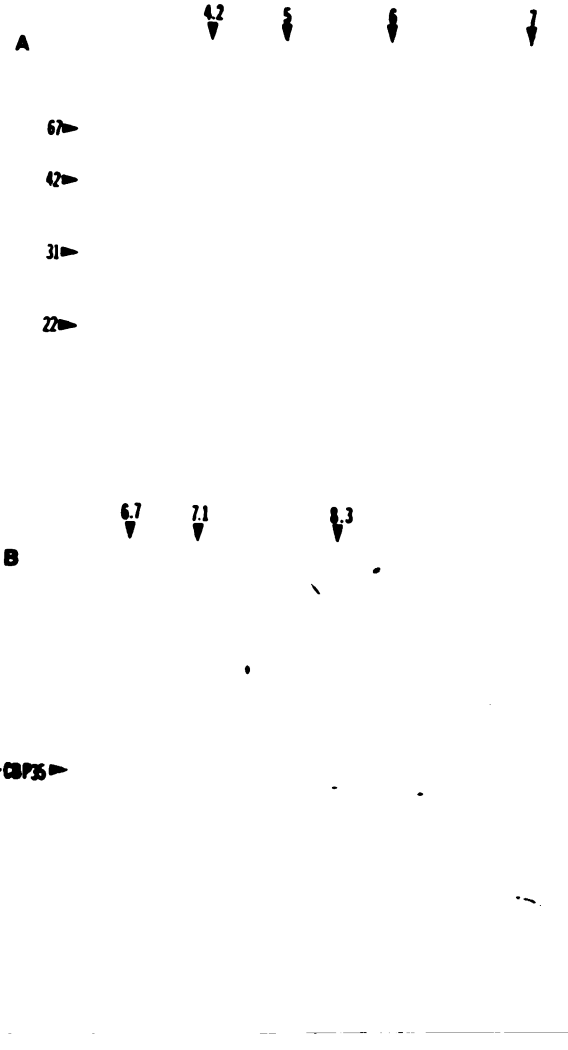
culture (Table II), the observed decrease in the amount of CBP35 per unit of protein must necessarily reflect a decrease in the CBP35 content per cell. Together, the immunofluorescence and immunoblotting results are consistent with the notion that as SL66 fibroblasts age *in vitro*, they lose the regulation of the expression of CBP35: down regulation upon approach to quiescence due to serum starvation and up regulation upon readdition of serum.

Identification of the Isoelectric Variants of CBP35 in Human Fibroblasts by Two-Dimensional Gel Electrophoresis

Previous studies (8) on recombinant CBP35 had identified the isoelectric point of the mouse polypeptide to be pI 8.7 by two-dimensional gel electrophoresis. Extracts of mouse 3T3 fibroblasts yielded two isoelectric species for CBP35, pI 8.7 and pI 8.2. We had identified the latter to be a derivative of the pI 8.7 polypeptide due to the addition of a single phosphate group. In these studies, two types of two-dimensional gel analysis were used: IEF and NEPHGE (8). This was done because certain proteins with basic pI values do not focus at the high pH end of the IEF gradient. On the other hand, the resolution of the NEPHGE analysis at the low pH end was often poor. Moreover, samples are usually loaded from the basic end for IEF and from the acidic end for NEPHGE. Because of these differences, both IEF and NEPHGE analyses were carried out in our initial search for the isoelectric variants of the CBP35 from SL66 cells.

Extracts of SL66 fibroblasts (passage 11) were subjected to electrophoresis on IEF and NEPHGE gels, followed by immunoblotting with anti-CBP35. On IEF gels, noimmunoreactive spots were observed (Figure 4A). Analysis of the same extract on

Figure 4: **Two-dimensional electrophoretic analysis of CBP35 in extracts of human SL66 fibroblasts on IEF (A) and NEPHGE (B) gels.** Extracts of SL66 cells (100 μg total protein) were electrophoresed and the protein was revealed by immunoblotting with rabbit anti-CBP35. The numbers at the top indicate the pH values of the ampholine gradient. The numbers at the left indicate the positions of migration of molecular weight markers. The position of migration of recombinant mouse CBP35 is shown as a reference for panel B.


4.2
5
6
7
A
67
42
31
22
6.7
7.1
8.3
B
rCBP35

NEPHGE gels revealed two spots, with pI values of 8.7 and 8.2 (Figure 4B). These results are identical to what were observed with extracts of mouse 3T3 cells (8). On the basis of that previous study, we infer that the pI 8.7 species represented the unmodified CBP35 polypeptide, while the pI 8.2 species represented the phosphorylated derivative.

It should be noted that the positions of migration of the pI 8.7 and 8.2 spots remained the same, relative to standards with known pI values (e.g. carbamylated glyceraldehyde 3-phosphate dehydrogenase, pI 8.3), irrespective of whether the NEPHGE gel was electrophoresed for 1, 3, or 6 hours. Therefore, although the value obtained from NEPHGE analysis may not actually represent an equilibrium isoelectric point, we will, nevertheless, refer to the pH in the gel to which a polypeptide migrates as its pI value.

Serum-starved SL66 fibroblasts of low passage (passage 11) yielded a single isoelectric species under NEPHGE analysis, pI 8.2 (Figure 5). Upon serum stimulation, the intensity of this spot increased ~ 5.9-fold (Table IV). In addition, the pI 8.7 species also appeared. Serum-starved SL66 fibroblasts of high passage (passage 35) showed a single spot (pI 8.2) on NEPHGE gels (Figure 5). The intensity of this spot was 5.5-fold greater than the corresponding spot in serum-starved cells of low passage. Moreover, the intensity of this pI 8.2 spot does not change appreciably upon serum stimulation in the old cells (Table IV). These results are consistent with the previous observations that the level of CBP35 in high passage SL66 fibroblasts was rather insensitive to serum/growth state modulation. Finally, there was no evidence of any pI 8.7 CBP35 species in serum-stimulated cultures of passage 35 human fibroblasts (Figure 5). This is in direct contrast to the results obtained with passage 11 human SL66 cells and with mouse 3T3 fibroblasts, in whichextracts of serum-stimulated cells show the pI 8.7 spot.

Figure 5: **Comparison by NEPHGE gels of the isoelectric variants of CBP35 in cultures of human SL66 fibroblasts synchronized by serum starvation (Q) and by serum stimulation (P).** P11, SL66 cells at passage 11; P35, SL66 cells at passage 35. Approximately 100 μg of each sample were electrophoresed and the protein was detected by immunoblotting with rabbit anti-CBP35. The positions of migration of the pI 8.2 and the pI 8.7 species are indicated at the top.

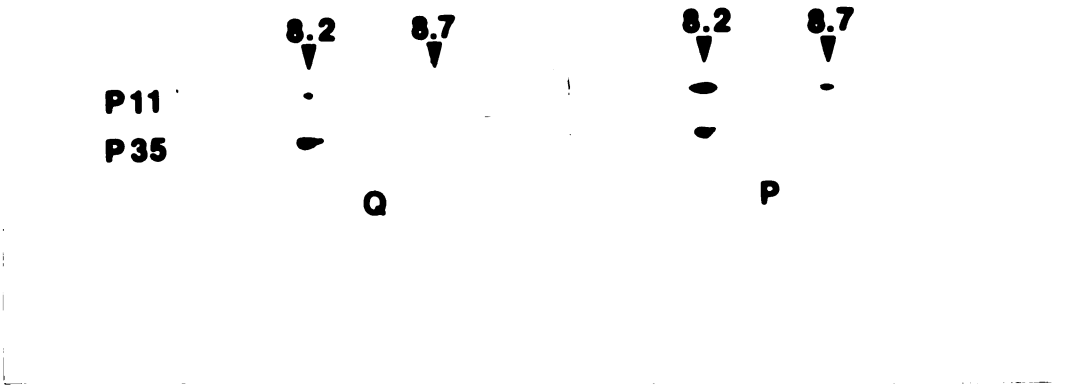

8.2
8.7
8.2
8.7
P11
P35
Q
P

Table IV. Levels of phosphorylated and unphosphorylated CBP35 in whole cell extracts of "young" versus "old" SL66 cells*

Extract	Serum-Starved (Q)		Serum-Stimulated (P)	
	pI 8.2	pI 8.7	pI 8.2	pI 8.7
P11	1.0	---	5.9	1.4
P35	5.5	---	4.4	---

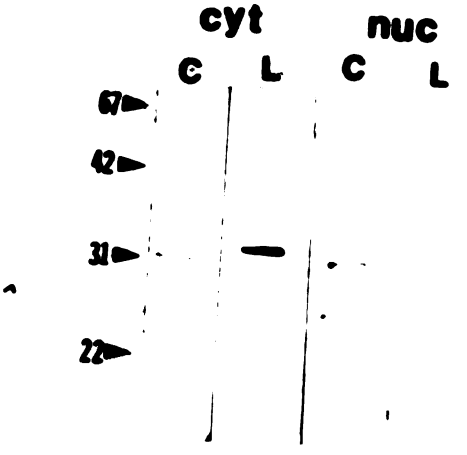
* The immunoblots shown in Figure 5 were subjected to densitometric scanning and the intensities of the pI 8.2 and pI 8.7 spots are expressed as arbitrary units of CBP35.

Nuclear Versus Cytoplasmic Distribution of the Isoelectric Variants

The cells of passage 11 and passage 35 cultures were also subjected to subcellular fractionation to yield a cytosol fraction (150,000 x g supernatant) and a nuclear pellet. The two subcellular fractions of passage 11 cultures were assayed by immunoblotting for CBP35 and for LDH, a cytosolic enzyme marker. The cytosol, which accounted for ~ 50% of the total protein, contained both CBP35 and LDH, as revealed by the immunoblot (Figure 6). Approximately 25% of the cellular protein was found in the nuclear pellet; the remainder of the protein was in membrane components that did not fractionate into either the 150,000 x g supernatant or the nuclear pellet. The nuclei yielded a M_r ~ 35,000 band in the anti-CBP35 blot, but showed no reactive bands whatsoever in the anti-LDH blot (Figure 6). These results, as well as marker enzyme and Hoechst dye binding assays, indicated that there appeared to be little or no contamination of the nuclear fraction by cytoplasmic components.

The cytosolic fraction from both serum-starved and serum-stimulated passage 11 cultures yielded only the pI 8.2 spot (Figure 7). The intensity of this spot in the cytosol of serum-stimulated cultures was 4.7-fold higher than that of the corresponding spot from serum-deprived cultures (Table V). This reflects the overall increase in the expression of the CBP35 polypeptide in proliferating cells. While the nuclei of serum-starved passage 11 cells showed only the pI 8.2 spot, the nuclei of serum-stimulated cells showed both the pI 8.2 as well as the pI 8.7 spots (Figure 7). The intensity of the pI 8.2 spot was about 4-fold higher in the nuclear fraction of serum-stimulated cultures than the corresponding fraction of quiescent cultures (Table V). Thus, the increased expression

Figure 6: **Detection of CBP35 and lactate dehydrogenase in the cytosol and nuclei fractions of human SL66 (passage 11) cells.** Approximately 20 μg of protein from the cytosol (cyt) and the nuclei (nuc) fractions were subjected to SDS-PAGE in each lane. C: Immunoblotting with rabbit anti-CBP35. L: Immunoblotting with rabbit anti-lactate dehydrogenase. The numbers indicate the positions of migration of molecular weight markers.


cyt
nuc
C
L
C
L
67
42
31
22

of CBP35 in proliferating cells is manifested most clearly in the nuclear fraction, with increases in both the pI 8.7 as well as in the pI 8.2 isoelectric variants.

The subcellular fractionation studies on passage 35 cells substantiate the conclusion obtained with whole cell extracts. First, there was no increase in the level of the pI 8.2 species between serum-deprived and serum-stimulated cultures, in either the cytosolic or the nuclear fractions (Table V). More strikingly, no pI 8.7 species was observed in the passage 35 cells (Figure 7). This conclusion was true in examining the cytoslic or nuclear fractions of serum-stimulated cultures, as well as the corresponding fractions of serum-starved cells.

Figure 7: **Comparison by NEPHGE gels of the isoelectric variants of CBP35 in serum-starved (Q) and serum-stimulated (P) cultures of human SL66 fibroblasts at passage 11 (P11) and passage 35 (P35).** Cyt, cytosol fraction; Nuc, nuclear pellet. Approximately 100 μg of each sample were electrophoresed and the protein was detected by immunoblotting with rabbit anti-CBP35. The positions of migration of the pI 8.2 and pI 8.7 species are indicated at the top.

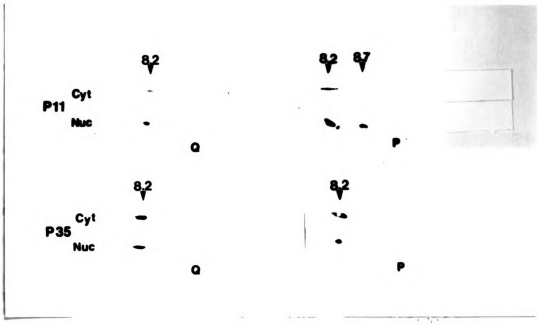

8.2
8.2
8.7
P11
Cyt
Nuc
Q
P
8.2
8.2
P35
Cyt
Nuc
Q
P

Table V. Levels of phosphorylated and unphosphorylated CBP35 in cytosol and nuclei of SL66 cells*

P11	Serum-Starved (Q)		Serum-Stimulated (P)	
	pI 8.2	pI 8.7	pI 8.2	pI 8.7
cytosol	1.0	---	4.7	---
nuclei	7.1	---	28	8.4
P35	Serum-Starved (Q)		Serum-Stimulated (P)	
	pI 8.2	pI 8.7	pI 8.2	pI 8.7
cytosol	1.4	---	1.7	---
nuclei	1.6	---	1.0	---

* The immunoblots shown in Figure 7 were subjected to densitometric scanning and the intensities of the pI 8.2 and pI 8.7 spots are expressed as arbitrary units of CBP35.

DISCUSSION

Using a highly specific antiserum directed against CBP35, we have analyzed the expression of this lectin at the protein level by immunofluorescence and by immunoblotting of extracts/subcellular fractions of human fibroblasts passaged *in vitro*. The following key observations have been documented: (a) The level of CBP35 in young (passage 11) SL66 fibroblasts is low in serum-deprived, quiescent cultures but increases dramatically (~ 9-fold) 17 hours after serum stimulation. A prominent aspect of this increase is the appearance of the unphosphorylated form of the CBP35 polypeptide in the nucleus of serum-stimulated cells. (b) Older (passage 31-35) SL66 fibroblasts fail to exhibit the correlation between the level of CBP35 and the proliferation state of the culture. The level of CBP35 remained high (no down regulation) in serum-starved passage 32 cells and serum addition resulted in a decrease rather than the expected increase in CBP35 expression. The unphosphorylated form of the CBP35 polypeptide was not observed in cultures of high passage SL66 cells.

These results are particularly interesting and need to be discussed in light of two additional, more recent observations. First, we have obtained preliminary evidence, by Northern blotting with a cDNA probe for CBP35 (3,9), that the level of the 1.3 kb mRNA for CBP35 was elevated upon serum stimulation of young SL66 fibroblasts, but not of the older high passage cells. Second, Seshadri and Campisi have compared the transcriptional expression of several genes upon serum stimulation of early passage and senescent human fetal lung (WI-38) fibroblasts (30). Although serum induced the mRNA for c-H-ras,

c-myc, and ornithine decarboxylase normally, it failed to induce the mRNA for the c-fos protooncogene in senescent cells.

Taken in this context, it may be important to emphasize several parallels in the regulation of expression of the genes for CBP35 and c-fos in mouse 3T3 fibroblasts. The mRNAs for both genes are elevated early upon serum addition to quiescent cultures. An increase in the level of the c-fos mRNA is seen within 15 minutes after stimulation (31,32); we have observed increases in CBP35 mRNA levels 30 minutes following serum addition (10). At least part of the increase in mRNA levels of c-fos and CBP35 is due to an increase in the rates of transcription of the two respective genes, as revealed by nuclear run-off assays (10,31). Moreover, the increased transcriptional expression of these genes was observed even when the stimulation of the cells was carried out in the presence of cycloheximide (10,33). Such observations lend support to the notion that the increased expression of the CBP35 and c-fos genes is a direct result of signals transduced by the binding of growth factors to their plasma membrane receptors, without the requirement of prior synthesis of other gene products. In the nucleotide sequence of the c-fos gene, a regulatory sequence designated SRE has been identified in the 5′ flanking region, some 300 nucleotides upstream from the site of transcription initiation (12). The SRE confers serum inducibility by binding specific transcription factors (serum response factor). Our recent analysis of the genomic sequence for CBP35 has revealed a candidate for a SRE-like structure (11). The sequence in the CBP35 gene, CCAATTAAGG, differs from the consensus SRE sequence, C-C-A-A/T-A-T-A/T-A/T-GG (12,13), at one position. An A residue in position 5 of the 10-nucleotide consensus is replaced by a T residue in the CBP35 gene.

Our present demonstration that senescent human fibroblasts fail to activate the expression of CBP35 adds another item to the list of similarities between the c-fos and CBP35 genes. Thus, these two genes are distinguished from other mitogen-stimulated and/or cell cycle-regulated genes, such as ornithine decarboxylase, thymidine kinase, replication dependent histone 3, c-myc, and β-actin (30,34,35). The latter is of particular interest because the β-actin gene also contains the SRE regulatory sequence and, like c-fos, is regulated by serum response factor (13). Although the addition of serum to senescent WI-38 fibroblasts results in a lower level of stimulation of the actin gene than in corresponding cells at early passage, there is, nevertheless, a clear elevation of transcription rate, as well as accumulated mRNA (30). This suggests that senescent cells are not deficient in serum response factor or in signals that activate it. On this basis, the failure of CBP35 and c-fos genes to respond to serum stimulation must be ascribed to a specific transcriptional repression mechanism.

It should be noted that the older (passage 31-35) SL66 cells not only fail to activate the CBP35 gene upon serum stimulation of serum-deprived cultures, they also appear to have lost the down regulation of CBP35 during serum starvation. When mouse 3T3 fibroblasts and low passage SL66 cells were deprived of serum, there is a drastic decrease in the level of CBP35 protein, as revealed by immunofluorescence and by immunoblotting. On the other hand, high passage SL66 fibroblasts starved of serum retain relatively high levels of CBP35, all of which exist in the phosphorylated (pI 8.2) form of the protein in the cytoplasm. Examination of the percent of CBP35 positive cells in intermediate passage (passage 17) and in late passage (passage 31) cells suggest that as the cultures age *in vitro*, a certain fraction of the cells depart from the normal

regulatory mechanism, including the down regulation of CBP35 protein levels. This results in about 50% of the SL66 cells to be considered positive for CBP35 in our immunofluorescence quantitation. Whether this portion of the cell population that has lost the regulation of CBP35 corresponds to the same population that has lost responsiveness to serum induction of cell proliferation remains to be determined.

REFERENCES

1. Roff, C.F., and Wang, J.L. (1983) J. Biol. Chem. 258, 10657-10663.
2. Crittenden, S.L., Roff, C.F., and Wang, J.L. (1984) Mol. Cell. Biol. 4, 1252-1259.
3. Jia, S., and Wang, J.L. (1988) J. Biol. Chem. 263, 6009-6011.
4. Drickamer, K. (1988) J. Biol. Chem. 263, 9557-9560.
5. Moutsatsos, I.K., Davis, J.M., and Wang, J.L. (1986) J. Cell Biol. 102, 477-483.
6. Laing, J.G., and Wang, J.L. (1988) Biochemistry 27, 5329-5334.
7. Moutsatsos, I.K., Wade, M., Schindler, M., and Wang, J.L. (1987) Proc. Natl. Acad. Sci. USA 84, 6452-6456.
8. Cowles, E.A., Agrwal, N., Anderson, R.L., and Wang, J.L. (1990) J. Biol. Chem., in press.
9. Jia, S., Mee, R.P., Morford, G., Agrwal, N., Voss, P.G., Moutsatsos, I.K., and Wang, J.L. (1987) Gene (Amst.) 60, 197-204.
10. Agrwal, N., Wang, J.L., and Voss, P.G. (1989) J. Biol. Chem. 264, 17236-17242.
11. Jia, S., and Wang, J.L. (1990) J. Biol. Chem., submitted.
12. Treisman, R. (1986) Cell 46, 567-574.
13. Gustafson, T.A., Miwa, T., Boxer, L.M., and Kedes, L. (1988) Mol. Cell Biol. 8, 4110-4119.
14. Roff, C.F., Rosevear, P.R., Wang, J.L., and Barker, R. (1983) Biochem. J. 211, 625-629.
15. Cowles, E.A., Moutsatsos, I.K., Wang, J.L., and Anderson, R.L. (1989) Exp. Gerontol. 24, 577-585.

16. Hayflick, L. (1965) Exp. Cell Res. 37, 614-636.

17. Cristofalo, V.J., and Sharf, B.B. (1973) Exp. Cell Res. 76, 419-427.

18. Drinkwater, N.R., Corner, R.C., McCormick, J.J., and Maher, V.M. (1982) Mutat. Res. 106, 277-287.

19. Cowles, E.A., Brauker, J.H., and Anderson, R.L. (1987) Exp. Cell Res. 168, 347-356.

20. Courtneidge, S.A., Levinson, A.D., and Bishop, J.M. (1980) Proc. Natl. Acad. Sci. USA 77, 3783-3787.

21. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

22. Kaplan, N.O., and Cahn, R.D. (1962) Proc. Natl. Acad. Sci. USA 48, 2123-2130.

23. Hay, A.J. (1971) Virology 60, 398-418.

24. Cesarone, C.F., Bolognesi, C., and Santi, L. (1979) Anal. Biochem. 100, 188-197.

25. O'Farell, P.H. (1975) J. Biol. Chem. 250, 4007-4021.

26. O'Farell, P.Z., Goodman, H.M., and O'Farell, P.H. (1977) Cell 12, 1133-1142.

27. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.

28. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.

29. Salk, D. (1982) Hum. Genet. 62, 1-15.

30. Seshadri, T., and Campisi, J. (1990) Science 247, 205-209.

31. Greenberg, M.E., and Ziff, E.B. (1984) Nature (Lond.) 311, 433-438.

32. Muller, R., Bravo, R., Burckhardt, J., and Curran, T. (1984) Nature (Lond.) 312, 716-721.

33. Lau, L.F., and Nathans, D. (1987) Proc. Natl. Acad. Sci. USA 84, 1182-1186.

34. Chang, Z.F., and Chen, K.Y. (1988) J. Biol. Chem. 263, 11431-11435.

35. Stewart, C.J., Ito, M., and Conrad, S.E. (1987) Mol. Cell Biol. 7, 1156-1163.