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Topical Review

Neutral Amino Acid Transport Systems in Animal Cells: Potential Targets of Oncogene Action and Regulators of Cellular Growth

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A. Introduction

Amino acid uptake by a tissue cell in a multicellular organism appears to be one of several determinants of growth rate. While a multiplicity of transport systems for amino acids exists for the uptake of these essential nutrients, only a few of these have been subject to careful and critical analysis (*see* section B). Of these systems, one particular system, the system A transporter, has been found to be regulated by a wide variety of external stimuli and conditions (section C), and in some cells, its activity correlates reasonably well with growth state (section D). System A is thus one of the few identified transport systems which appears to be a target of protooncogene and oncogene action and regulator of cellular growth (section E). In this review, the evidence for this postulate is presented and evaluated.

B. A Multiplicity of Amino Acid Transporters in Animal Cells

1. Neutral Amino Acid Transporters

Properties of some of the best characterized neutral amino acid transport systems in tissue cells are summarized in Table 1. The subcellular localizations and regulatory characteristics of these systems in the well-characterized dog kidney epithelial cell line, MDCK, are also presented. The amino acid transporters show overlapping specificities and have been characterized both biochemically and genetically in a number of cell types and cell lines. (For recent reviews, *see* Shotwell, Kilberg & Oxender, 1983; Shotwell & Oxender, 1983; Englesberg & Moffett, 1986; Colarini & Oxender, 1987.) System A was initially characterized in Ehrlich ascites cells in 1963 by Oxender and Christensen (*see* Colarini & Oxender, 1987, for a recent review). It preferentially transports short, straight-chain amino acids such as alanine, glycine, proline and the system A-specific, nonmetabolizable amino acid analog 2-(methylamino)-isobutyrate (MeAIB) (Bass et al., 1981). System A has been operationally defined as the system which is responsible for MeAIB-inhibitable amino acid uptake in the presence of a sodium gradient. System A has been found in many vertebrate cell lines and is localized predominantly to the basolateral membrane in confluent MDCK cells (Lever, Kennedy & Vasan, 1984; Boerner et al., 1986). It is inactive at pH 6.0. Lever et al. (1984) have measured a 1 : 1 stoichiometry for sodium ion to amino acid transported using membrane vesicles of MDCK cells. Adaptive regulation (also termed starvation-induced transport or repression/derepression), hormonal regulation, transinhibition, and responses of system A to cellular transformation will all be discussed in sections C and D.

System ASC is also sodium-dependent and transports alanine, serine, cysteine, and threonine most efficiently. It does not recognize N-methylated amino acid derivatives. System ASC functions over a broad pH range with only 20% loss of maximal activity when the pH is shifted from 7.4 to 6.0, and it is localized to the basolateral membrane of confluent MDCK cells (Boerner et al., 1986). System ASC is functionally defined as that system responsible for the sodium gradient-dependent amino acid uptake not inhibited by high concentrations of MeAIB. No model nonmetabolizable substrate exists for system ASC determination, and accurate assignment of activity is complicated by the presence of other sodium-dependent systems.

Key Words growth regulation · protein kinases · nutrient transport · tissue culture cells · targets of oncogene action · regulation

Table 1. Properties of well-characterized transport systems for neutral amino acids[a]

System:	A	ASC	L	G
1) Membrane localization:	Basolateral	Basolateral	Basolateral	Apical
2) Cation specificity:	Sodium	Sodium	Hydrogen(?)	Sodium
3) Substrate specificities:	Short, polar, straight chain amino acids; MeAIB	Alanine, serine, cysteine, and threonine;	Branched chain or aromatic amino acids; BCH	General
4) Regulation by:				
a) Prostaglandin E_1:	inhibits	no effect	no effect	stimulates
Insulin:	stimulates	stimulates	stimulates	little effect
b) Amino acid availability:	inhibits	no effect	little effect	little effect
c) Confluency:	inhibits	inhibits	little effect	stimulates
d) Transformation:	stimulates	no effect	inhibits slightly	no effect
e) Trans-affect:	inhibits	stimulates	stimulates	not known

[a] Cellular localizations and regulatory properties of these transport systems in the dog kidney epithelial cell line, MDCK, were determined as described (Boerner & Saier, 1982*a*; 1985*a*,*b*; Boerner et al., 1986).

To eliminate the possibility of transport by sodium-dependent systems other than system A, studies with the CHO cell line have used serine as a specific inhibitor of system ASC. In the presence of MeAIB, system ASC uptake is defined as the fraction of sodium-dependent accumulation which is blocked by high concentrations of serine (Bass et al., 1981). System ASC was first shown to be present in Ehrlich ascites cells, but in recent years it has been found in virtually every animal cell line examined (Christensen, Liang & Archer, 1967). System ASC appears to be relatively insensitive to adaptive regulation, hormonal control and transformation, but it is subject to transstimulation (Table 1).

System L preferentially catalyzes the uptake of branched-chain and aromatic amino acids by a sodium-independent mechanism. The nonmetabolizable analog, 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH), is an ideal and specific substrate. System L has been localized to the basolateral membrane in epithelial cells (Boerner et al., 1986), and it appears to be ubiquitous in eukaryotic cells. System L does respond to adaptive regulation upon starvation for leucine (and to a lesser extent for isoleucine, phenylalanine, or valine) in CHO cells (Moreno, Lobaton & Oxender, 1985).

Many other amino acid-specific as well as general transport systems have recently been characterized. System P is a sodium-dependent system catalyzing the uptake of proline into CHO cells (Moffett et al., 1983). System N, specific for glutamine, asparagine and histidine, was initially characterized in rat hepatocytes. It was found to be subject to adaptive regulation but insensitive to hormonal control (Kilberg, Handlogten & Christensen, 1980). System β, found in rat hepatocytes and Ehrlich ascites cells, transports the naturally occurring β-amino acid, taurine, in a sodium-dependent manner (Christensen, 1964). System Gly accumulates glycine by a sodium-dependent mechanism which is not subject to adaptive regulation or hormonal control (Vidaver, 1964; Christensen & Handlogten, 1981). Another glycine uptake system has been characterized in renal brush-border vesicles (Rajendran et al., 1987). This system is driven by an inward proton gradient and transports glycine, proline, hydroxyproline, sarcosine, and β-alanine. The sodium-independent system, asc, found in horse erythrocytes and pancreatic epithelium, transports alanine, serine, and cysteine and is subject to stimulation by insulin (Fincham, Mason & Young, 1985; Norman & Mann, 1987). System G has been characterized in MDCK cells as a sodium-dependent "general" amino acid transporter localized to the apical membrane of these cells (Boerner et al., 1986). Its synthesis appears to be cAMP-dependent (D. Dean and M.H. Saier, Jr., *unpublished results*).

Lysosomes are a major intracellular site for macromolecular degradation. Several amino acid transport systems have recently been characterized in purified human fibroblast lysosomes (Pisoni et al., 1987). These systems appear to be sodium independent and have overlapping specificities. An ATP-dependent cystine transporter has also been reported in human lymphoblast lysosomes (Jonas et al., 1982).

2. System A Regulation—A Brief Summary

System A regulation has been proposed to occur at the level of transcription as well as by carrier modifi-

cation (Guidotti et al., 1979). System A activity is responsive to the cellular energy levels as well as exogenous amino acid availability. In hepatic cells, the best substrates for system A are also the best substrates for gluconeogenesis (Kilberg, Barber & Handlogten, 1985). The transport of these amino acids has been proposed to be the rate-limiting step in their utilization and thus a natural site of regulation (Kilberg, 1986). Since changes in system A activity apparently reflect changes in the environment, system A responds to hormonal stimulation, cellular growth state, amino acid availability, and degree of transformation. The regulation of system A will be summarized briefly in this section and discussed in much greater detail in section C.

Hormones coordinate the metabolic processes of organisms. Exposure of CHO-K1 cells to insulin results in a three- to fourfold stimulation of system A transport in a defined medium (Mendiaz et al., 1986). Stimulation by insulin has been shown to be dependent on both RNA and protein synthesis. Exposure of rat liver parenchymal cells to insulin results in an initial rapid increase of system A activity followed by a slower secondary increase (Kletzien et al., 1976). Studies involving membrane vesicles derived from 3T3 mouse fibroblasts implicated cAMP and the cAMP-dependent protein kinase as a negative regulator of system A transport (Nilson-Hamilton & Hamilton, 1979). However, convincing evidence for a correlation between cAMP levels and system A transport in any cell line is at present lacking, and a stimulatory effect of glucagon, which also stimulates cyclic AMP production, increased system A activity in liver cells (Kilberg & Neuhaus, 1977). Recent work involving the use of MDCK cells grown in a defined medium showed a requirement for low concentrations of prostaglandin E_1 for maximal system A activity, but high concentrations of this hormone lowered the activity in the presence of repressive amino acids (Boerner & Saier, 1985*a*,*b*). These same publications reported results which led to the suggestion that stimulation by insulin involved synthesis of new carriers as well as inhibition of carrier inactivation. System A activity increases in rat hepatocytes upon exposure of the cells to glucagon. Glucagon stimulation appears to be dependent on the availability of intracellular, mobilizable calcium (Kelley, Evanson & Potter, 1980).

As cells approach confluency in tissue culture, the rate of cellular metabolism decreases. Changes in MDCK cell growth rate consistently correlate with changes in system A activity in the presence of repressive amino acids (Boerner & Saier, 1982*a*,*b*; *see* section D). Several factors appear to be involved in this repression/depression phenomenon. First, intracellular concentrations of amino acids increase when cells reach confluency. High intracellular levels of amino acids have been shown to repress system A activity in an RNA and protein synthesis-dependent manner. Handlogten and Kilberg (1984) have proposed that intracellular amino acids exert their effects via a "transport inactivating protein" (TIP) which belongs to a class of proteins whose mRNA's lack poly (A) tails. This observation is consistent with TIP's presumed transcriptional regulatory function. Recently, Dawson and Cook (1987) have described a correlation between system A activity and protein kinase C localization. This protein kinase shifts from the membrane to the cytosol as the cells approach confluency but shifts back to the membrane, regardless of growth state, upon addition of 12-0-tetradecanoylphorbol-13-acetate (TPA). System A activity in confluent cells responds in two ways to the addition of TPA. An initial, rapid but transient increase in transport activity may result from the phosphorylation of the carrier or a regulatory protein controlling its activity. A second increase in transport activity becomes noticeable 30 min after exposure to TPA and could be the result of increased transcription of the gene encoding the transport protein.

System A activity also reflects the availability of exogenous amino acids. Removal of amino acids from the growth medium leads to derepression of system A within 4–5 hr. Repression begins within 30 min after the addition of certain amino acids in a protein synthesis-dependent manner. However, transport of an amino acid by system A does not necessarily correlate with its repressive capacity. Inhibitors of alanine uptake such as diaminobutyrate, phenylalanine, α-ketoglutarate, pyroglutamate, isoleucine, and valine have no effect on derepression, whereas β-alanine, histidine, and hydroxyproline, which do not inhibit proline uptake via system A, strongly repress. Repression occurs with a half-life of one and a half hours (Fafournoux, Rémésy & Demigné, 1985; Moffett & Englesberg, 1986). Transinhibition, thought to result from a direct action of the intracellular amino acid on the carrier, is also observed when the uptake of a radioactive amino acid is being followed.

Transformation of a cell line generally leads to an increase in system A activity without a corresponding increase in the activities of other amino acid carriers (Foster & Pardee, 1969; Isselbacher, 1972). Using the MDCK and the chemically transformed MDCK-T_1 cell lines, Boerner and Saier (1982*a*) demonstrated that the MDCK-T_1 cells exhibited increased system A activity. These cells were resistant to repression of transport activity by external amino acids although they were still subject to transinhibition. The transport of AIB in 3T3 cells and a virally transformed 3T3 cell line (Py3T3)

showed similar differences (Foster & Pardee, 1969). The rate of AIB accumulation, probably via system A, decreased by 30% as normal 3T3 cells became confluent whereas the Py3T3 cell line showed no difference between confluent and nonconfluent growth states. Py3T3 cells generally showed a twofold increase in AIB transport over the nonconfluent 3T3 cells.

3. Postulated Regulatory Mechanism Controlling System L Synthesis in CHO Cells

The regulation of system L is genetically well characterized in CHO cells. Reduction in the concentration of leucine to less than 10 μM results in a three- to fourfold increase in system L activity (Moreno et al., 1985). Starvation for most other amino acids has no effect on system L activity. However, removal of isoleucine, phenylalanine or valine results in half-maximal derepression (Moreno et al., 1985). Growth of a temperature-sensitive $tRNA^{leu}$ synthase mutant (CHOtsH1) at marginally permissive growth temperatures results in a two- to threefold increase in system L activity (Moore, Jayme & Oxender, 1977). The increase in activity is the result of an increase in the V_{max} with no change in the K_m, perhaps indicating an increase in the number of transporters rather than carrier modification. Temperature-dependent stimulation was prevented by cycloheximide but not by actinomycin D suggesting that regulation is at the level of translation (Shotwell et al., 1982).

Suppression of the temperature-sensitive phenotype with cell fusions to human leukocytes has localized the $tRNA^{leu}$ synthase gene to chromosome 5 and the gene encoding the System L carrier protein to chromosome 20 (Lobaton, Moreno & Oxender, 1984). These studies are in agreement with the postulate of Moore et al. (1977) who suggested that the regulation of system L is dependent on the concentration of charged $tRNA^{leu}$ molecules. When cell growth is inhibited by cycloheximide, or when cells reach confluency, internal amino acid concentrations increase, which is believed to cause system A activity to be inhibited and system L activity to be stimulated (Oxender, Lee & Cecchini, 1977). Stimulation of uptake by internal amino acids is characteristic of an exchange-type mechanism. Growth of the cells in a rich medium and subsequent exposure to an amino acid-free medium results in the rapid depletion of cytoplasmic system L amino acids as compared to substrates of system A. System L has been found to be relatively unresponsive to hormone stimulation and growth state in MDCK cells (Boerner & Saier, 1982*a*).

4. Noncoordinate Regulation of Neutral Amino Acid Transport

The results summarized above suggest that the different amino acid carriers in animal cells are regulated by quite distinct mechanisms. In accordance with this postulate, studies with the MDCK cells have revealed that systems A, ASC, and L are noncoordinately regulated (Boerner & Saier, 1982*a*; *see* next section).

C. Multiple Mechanisms Regulating System A

1. Direct Regulation of System A Activity by Carrier Modification

At present little is known regarding the molecular properties of the system A carrier protein. Several studies have indicated that the carrier may have an N-linked oligosaccharide moiety and free sulfhydryl groups, but the protein itself has yet to be identified. In a study by Dudeck et al. (1987), it was found that there are inherent differences between system A carriers which are present in normal and transformed liver cells. These particular experiments were performed with isolated membrane vesicles and reconstituted proteoliposomes to ensure that the differences observed were not due to differing cellular structure, metabolic activity, or intracellular sulfhydryl modification in the two cell types.

Two sulfhydryl-modifying reagents, N-ethylmaleimide (NEM) and *p*-chloromercuribenzene sulfonate (pCMBS) were found to inhibit transport of the system A substrate, 2-aminoisobutyrate (AIB), in rat liver hepatocytes. System A transport was also inactivated employing pCMBS in cultured rat H4 hepatoma cells, but transport in these transformed cells was unaffected by treatment with NEM. pCMBS decreased transport 80–90% in a concentration-dependent manner in hepatoma-derived vesicles, and this inactivation could be prevented by addition of amino acid substrates of system A. Following removal of the protective amino acid, inactivation occurred upon addition of the sulfhydryl-specific reagents. This observation suggested that one or more cysteine residues became less reactive in the presence of a system A substrate. Substrate-dependent protection from pCMBS inactivation was not observed in vesicles from normal hepatocytes. These results suggest that the protein responsible for system A transport may exhibit structural differences in various cell types. It should be noted that MeAIB, but not AIB, is a specific substrate of system A in many cell

types including liver cells (Kilberg & Neuhaus, 1977; Boerner & Saier, 1982*a*). Consequently, although hepatoma cells appear to lack system ASC, the interpretation of the study of Dudeck et al. (1987), which resulted from studies only with AIB, must be considered somewhat equivocal.

Dawson and Cook (1987, 1988) have observed a biphasic temporal response of Na^+-dependent system A transport to tumor-promoting phorbol esters in pig kidney epithelial cells, LLC-PK_1. 12-O-Tetradecanoyl phorbol 13-acetate, TPA, activates protein kinase C, an enzyme regulated by the phosphoinositide cycle important in cellular responses to certain hormonal agents. The initial rapid response to TPA was found to occur in 5–7 min, returning to near-control levels by 20 min. The second response, beginning after 30 min or more, continued to rise for more than 3 hr. The early, transient, stimulatory phase could be inhibited by sphingosine, an inhibitor of protein kinase C, while the subsequent decrease in system A activity could be delayed by addition of sodium azide, a protein-phosphatase inhibitor. These results suggest that the early response to TPA involves the phosphorylation either of the system A carrier itself or of some other protein which acts to regulate its activity. With the data presently available, these two possibilities cannot be distinguished. The relationship of these findings to those of Dudeck et al. (1987) is not known. The second response presumably involves gene transcription and *de novo* protein synthesis.

A biphasic kinetic response to substrate concentration has been demonstrated in Madin-Darby canine kidney (MDCK) cells (Boerner & Saier, 1985*a*,*b*).[1,2] As for the results of Dawson and Cook (1987) and Dudeck et al. (1987), it is not clear whether two system A structural genes encoding kinetically distinguishable carrier proteins, or a single system A structural gene encoding a carrier which can be modified by a post-translational mechanism is responsible for the results. The relationship, if any, between the two populations of presumed system A carriers in each of the three cell types is not known.

[1] P. Boerner and M.H. Saier, Jr. Effects of 5-azacytidine, sodium butyrate, and phorbol esters on amino acid transport system A in the kidney epithelial cell line, MDCK: Evidence for more than one mechanism of regulation. *J. Cell. Physiol.* (*in press*).

[2] P. Boerner and M.H. Saier, Jr. Isolation and characterization of MeAIB-resistant variants from a chemically induced oncogenic transformant of canine kidney epithelial cells (MDCK). *Somatic Cell Molec. Genet.* (*submitted*).

2. Adaptive Regulation of System A Synthesis

Animal cells adapt to fluctuations in extracellular amino acid availability by adjusting the activities of certain transport systems. In numerous cell types it has been shown that transport of neutral amino acids via system A can be induced by incubation of cells in amino acid-free media. This phenomenon has been referred to as "starvation-induced transport enhancement" or "adaptive regulation." Starvation-induced transport of amino acids by system A is characterized by an increase in the V_{max} for uptake. Although several early studies indicated that this derepression phenomenon occurred without any apparent change in K_m, K_m changes in the MDCK cells have now been documented (Boerner & Saier, 1985*a*,*b*).[1,2] Increased uptake due to adaptation is not a result of release from the phenomenon of transinhibition since the same derepressive effect is produced in cells depleted of amino acids prior to experimental measurements (Moffett & Englesberg, 1986). As demonstrated by studies employing inhibitors of transcription and translation, actinomycin D and cycloheximide, respectively, both RNA and protein synthesis are required for adaptive regulation (Shotwell et al., 1983). Gazzola and colleagues (1981) have shown that actinomycin is ineffective at blocking derepression when added after a 90-min starvation period. Rates of transcription therefore seem to increase shortly after initiation of amino acid deprivation, and if the mechanism of adaptive regulation involves a change in transcription of a system A structural gene, the resultant messenger RNA may be fairly stable. Alternatively, the requisite transcriptional event might involve expression of a gene encoding a system A modifying enzyme or protein, in which case a stable messenger RNA need not be postulated because the enzyme produced would be expected to be much more stable than the mRNA. The complex of glycosylating enzymes might be this "modifying enzyme" (*see below*). Inhibition of protein synthesis prevents any further increase in transport regardless of whether cycloheximide is added when cells are deprived of amino acids, or after an extended period (in excess of 90 min). Tunicamycin, which inhibits asparagine-linked glycoprotein biosynthesis, also blocks adaptive regulation in rat hepatocytes (Barber et al., 1983; Kilberg, 1986). These last results suggest that the system A-associated protein synthesized in response to amino acid starvation is a glycoprotein, and that the carbohydrate moiety is in some way required for activity, stability, or membrane insertion of the carrier.

Reversal of starvation-induced transport en-

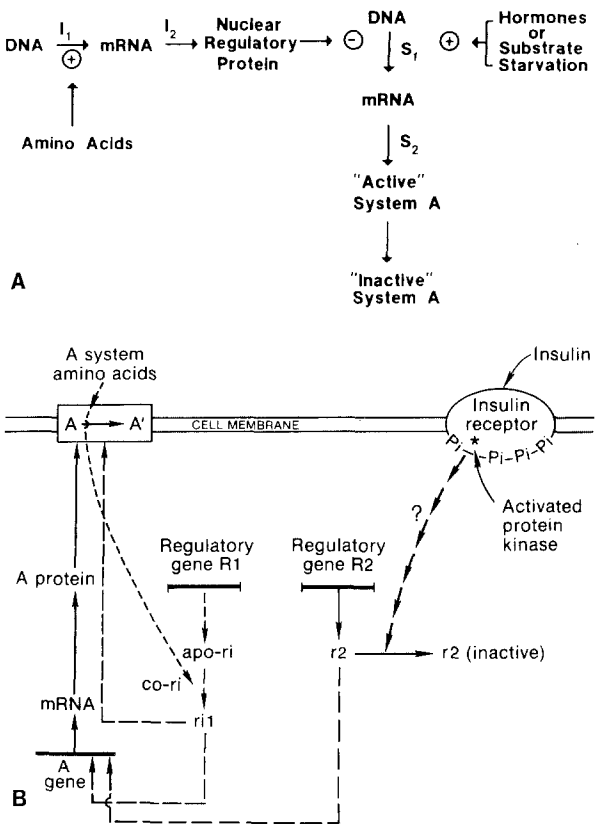

Fig. 1. Models of system A regulation. (*A*) The Kilberg model for adaptive regulation of system A activity in rat liver. I_1 and I_2 represent the transcriptional and translational steps, respectively, in the production of a nuclear regulatory protein (repressor) which negatively controls transcription of the system A structural gene. Step 1, transcription, and step 2, translation of the system A mRNA, gives rise to the active carrier which subsequently can be inactivated. (Reproduced from Kilberg, 1986, with permission). (*B*) The Englesberg model for the regulation of system A activity in CHO cells. R1 and R2 represent two regulatory genes the products of which are postulated to control system A synthesis. R1 encodes an aporepressor/inactivator (apo-ri) that is in equilibrium with a repressor/inactivator (ri1). Various amino acids can function either as corepressors or coinactivators or both (co-ris). The binding of a co-ri to the apo-ri shifts equilibrium toward the ri1 form. ri1 binds to a controlling region (operator) of the system A structural gene, gene A, thereby preventing transcription. Superimposed on the regulation by amino acid is the control by insulin which somehow functions through regulatory gene, R2, or its gene product, a constitutive repressor, r2. *See* text for details of the model. (Reproduced from Kilberg, 1986, with permission)

hancement occurs upon readdition of repressive amino acids to the growth medium. Transport is transinhibited, synthesis is repressed, and the carrier appears to be irreversibly inactivated. Most but not all of the system A amino acid substrates are individually capable of repressing system A synthesis in the absence of other amino acids (Moffett & Englesberg, 1986). Nonmetabolizable system A analogues, AIB and MeAIB, are particularly effective, demonstrating that substrate metabolism is not required for repression. As noted above, the adaptive response to individual amino acids required *de novo* RNA and protein synthesis in both chick embryo fibroblasts and cultured human fibroblasts (Shotwell et al., 1983). Other amino acids not generally transported by system A, including β-alanine, histidine and hydroxyproline, can also inhibit the starvation-promoted increase in activity (Englesberg & Moffett, 1986). The spectrum of repressive amino acids has also been determined for the MDCK cell system, and in general, repression does not correlate with transport (Boerner & Saier, 1985*a*). These results, in agreement with those of Moffett and Englesberg (1986), argue against a model of regulation involving direct participation of the system A transporter. The model put forth by Gazzola et al. (1981), postulating that substrate binding to the system A transporter is essential for repression and inactivation, is therefore probably incorrect.

The requirement of messenger RNA synthesis for derepression has been researched in greater detail using specific inhibitors of poly(A) polymerase: cordycepin and adenine-9-β-D-arabinopyranoside. Both inhibitors completely blocked derepression but were without effect on system A repression (Kilberg, 1986). These observations led to two alternative postulates. First, the mRNA, encoding the system A-associated glycoprotein which is required for increased transport in response to amino acid deprivation, may possess a poly(A) extension, or second, the mRNA encoding the protein(s) responsible for amino acid-dependent repression may belong to a small mRNA class which lacks poly(A) extensions (Kilberg, 1986). Among members of this class are the DNA-binding histones some of which are thought to be involved, directly or indirectly, in regulating gene expression. These observations and postulates gave rise to one of the two models for adaptive regulation described in the next section.

3. Proposed Mechanisms of System A Transcriptional Regulation

Based on data from several laboratories concerning adaptive regulation, two molecular models have been put forth. Kilberg has proposed the model summarized in Fig. 1(*A*). This model assumes that the rate of inactivation of system A is (a) continuous, (b) high, and (c) independent of substrate concentration. Synthesis (steps S_1 and S_2) increases in the absence of extracellular amino acids. When S_2

exceeds the rate of inactivation, then increased transport activity is observed. Repression occurs when specific amino acids reduce transcription of the gene for the system A-associated glycoprotein. This model postulates that an intracellular amino acid-binding protein senses elevated levels of cytoplasmic amino acids. This binding protein promotes initiation of the synthesis of the poly(A)-deficient mRNA (step I_1) that codes for a nuclear regulatory protein which acts to negatively regulate expression of the system A gene. This protein is therefore the "sensor" which gives rise to amino acid-dependent repression.

An alternative model for system A adaptive regulation has been proposed by Englesberg and Moffett (1986) based on their work with the CHO-K1 cells. Figure 1(*B*) summarizes this more complicated model. This model suggests that expression of the system A genetic apparatus is controlled by at least two regulatory genes, R1 and R2. Gene R1 produces an apo-repressor-inactivator (apo-ri) which is in equilibrium with a repressor-inactivator (ri1). Apo-ri is converted to ri1 when a repressive amino acid is bound to the active site of this DNA binding protein. Gene R2 produces a constitutive repressor (r2). r2 and ri1 both regulate expression of gene A, the structural gene encoding the system A carrier.

When cells are exposed to insulin, the hormone binds to its receptor, and the receptor-associated, tyrosine-specific protein kinase is activated. In some unspecified way this action is postulated to regulate system A by neutralizing r2. In the derepressed state, that is, under conditions of amino acid starvation, equilibrium between apo-ri and ri1 is shifted towards apo-ri. The rate of A gene mRNA synthesis therefore increases, and additional system A carrier protein is produced. Addition of amino acids shifts the equilibrium toward ri1. Most system A substrates generally act both as co-repressors and co-inactivators (or co-repressor-inactivators; co-ris) and regulate the shift toward ri1 by binding the regulatory protein. Specific amino acid effectors also determine whether the altered molecule acts as a repressor (r) or a repressor-inactivator (ri). The repressor prevents further synthesis of system A carrier mRNA by reacting with the controlling site of the A gene. It should be noted that there is little evidence for a single protein functioning both as the repressor and the inactivator.

Gene R1 may play a role in the regulation of two additional amino acid transport systems, ASC and P. However, most of the evidence supporting the model comes from experiments involving the genetic analysis of system A regulation in CHO cells.

4. Chinese Hamster Ovary (CHO) Mutant Lines Defective for System A Expression

Moffett and Englesberg (1986) have isolated mutant CHO-K1 cells defective for system A taking advantage of the defect in proline biosynthesis which renders these cells dependent on proline for growth. Alanine-resistant mutants were selected by allowing growth on plates containing inhibitory concentrations of alanine. For wild-type CHO cells, such concentrations of alanine are toxic because alanine inhibits uptake of proline. Mutants thus obtained show increased transport of proline.

One of the mutants, Ala^r2, when compared with the parental strain, showed a twofold increase in the basal repressed level of transport and in the V_{max} of proline transport, but it showed no change in the K_m for proline. Ala^r4 showed a fivefold increase in the V_{max} with no change in K_m, and system A activity was not further derepressible. Membrane vesicles derived from this mutant showed increased activity over the parental strain under identical conditions of assay (Moffett et al., 1987). Though system A in this mutant could be transinhibited, it could not be irreversibly inactivated. This last result established that transinhibition and irreversible inactivation are independent phenomena.

Ala^r2 and Ala^r4 do not complement one another, showing that the mutations resulting in these partially and fully constitutive phenotypes reside within the same gene. However, when these mutant cells were fused to normal CHO cells that show adaptive regulation of system A, the hybrid cell lines were able to regulate system A transport activity. Therefore some *trans*-acting factor from the parental line can regulate the transport activity of the mutant.

Englesberg and Moffett (1986) proposed that the increased system A activity in these mutants was caused by a mutation in the regulatory gene, R1, controlling expression of the system A structural gene, rather than in the system A structural gene itself. The latter type of mutant would be expected to be co-dominant and would frequently exhibit altered K_m values. The mutants can be explained as follows. Since system A expression in Ala^r4 is constitutive (i.e. cannot be repressed), apo-ri can no longer be converted to ri. Thus, no inactivation is seen when amino acids (co-ris) are introduced into the medium. In Ala^r2 the apo-ri has reduced affinity for co-ris, and equilibrium is therefore shifted toward apo-ri, away from ri1, giving rise to less ri1 in the absence of a co-ri. Moffett and Englesberg therefore concluded that system A is regulated by cytoplasmic amino acid effectors employing a negative control mechanism.

Ala^{r}4-H3.9, a mutant derived from Ala^{r}4, possessed a V_{max} of proline transport through system A six times that of the Ala^{r}4 cells. SDS-PAGE analysis of endoplasmic reticular membranes and of plasma membrane vesicles possessing active system A transport in the Ala^{r}4-H3.9 and wild-type CHO-K1 cells, showed increased amounts of two protein bands (62–66 and 29 kilodalton) in the mutant relative to the parental cells. Therefore, the Ala^{r}4-H3.9 mutation may give rise to amplification of the system A transporter structural gene (Moffett et al., 1988).

Studies involving another constitutive mutant, Ala^{r}6, support the conclusion that this mutant resulted from a mutation in the regulatory gene, R2 (Moffett et al., 1987). In conjunction with the product of the regulatory gene, R1, the product of the R2 gene is believed to act negatively to control expression of the system A structural gene. When grown under repressive conditions, system A in the Ala^{r}6 mutant cells demonstrated characteristics similar to those of a derepressed parental culture or the Ala^{r}4 mutant, the constitutive mutant thought to result from a defect in the R1 gene. However, in contrast to the situation with the Ala^{r}4 mutant, system A is further derepressible in the Ala^{r}6 mutant. Appropriate crosses involving Ala^{r}6 demonstrate that this mutant is recessive to the wild type and complements Ala^{r}4. In addition, although insulin stimulates system A transport in normal CHO-K1 cells, the mutant, Ala^{r}6 is unresponsive to insulin. This observation suggests that in wild-type cells insulin stimulation of system A activity may result from insulin-promoted inactivation of the R2 repressor (Moffett et al., 1987).

5. Regulation of System A Synthesis in Kidney Epithelial Cell Lines

Two kidney epithelial cell lines have been examined with respect to amino acid transport regulation, the LLC-PK$_1$ and MDCK lines. While the former line is believed to have arisen from the proximal tubule of pig kidney (Chuman et al., 1982) the latter probably arose from the distal tubule of dog kidney (McRoberts, Taub & Saier, 1981). To the extent that parallel studies have been conducted, regulation of system A synthesis and/or activation in these two lines appears to respond to physiological conditions and pharmacological agents in qualitatively similar ways (Amsler & Cook, 1982; Amsler, Shaffer & Cook, 1983; Boerner & Saier, 1982*a*,*b*; 1985*a*,*b*;[1-3] Dawson & Cook, 1987, 1988). Both of these polarized cells in monolayer cultures possess basolateral system A carriers which exhibit high activity during rapid growth but decreased activity as cells approach confluency. Only part of this depression in activity can be attributed to a decrease in the electrochemical gradient for Na^+ which provides the driving force for uptake. In both cell types the tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA) prevents the loss of activity which results upon attainment of the confluent state. In both cell types amino acid starvation results in derepression of system A synthesis, probably by the same mechanism as described for other cell lines in section C.2. Thus, derepression is dependent on RNA and protein synthesis, and repressive amino acids are usually, but not always substrates of system A. The derepression phenomenon presumably serves to compensate for variations in the availability of external amino acids so that cytoplasmic homeostasis can be maintained.

Dawson and Cook (1987) have correlated the system A response to TPA with the distribution of protein kinase C between membrane-associated and cytosolic forms of the enzyme. Protein kinase C is a Ca^{2+}-dependent enzyme which is activated by diacyl glycerols, or artificially, by TPA. Cellular exposure to TPA presumably bypasses the normal physiological process by which diacylglycerols are endogenously generated from phospholipids in response to external stimuli (Kikkawa & Nishizuka, 1986). The activity of protein kinase C is enhanced by this membrane association. This fact appears to be relevant to growth regulation as the activity is generally associated with the particulate fraction of extracts derived from rapidly growing or transformed cells, while it appears soluble in extracts derived from normal, quiescent cells (Adamo et al., 1986). In the studies of Dawson and Cook (1987), precisely this observation was confirmed for the LLC-PK$_1$ cells, and addition of TPA to post-confluent cells induced a shift of activity from the cytosolic fraction to the membrane fraction. This shift in protein kinase C localization correlated with the elevation of system A activity in intact cells. Since both the increase in system A activity and the shift of protein kinase C to its membrane-associated form were blocked by the inhibitor of microfilament function, cytochalasin B, but the protein synthesis inhibitor, cycloheximide, blocked only the former process, it was suggested that protein kinase C redistribution represents just one essential step either in the transcriptional activation of the system A structural gene or of a system A regulatory gene (Dawson & Cook, 1987). It is not known if amino acid starvation promotes association of protein kinase C with the membrane. These considerations

[3] P. Boerner and M.H. Saier, Jr. The influence of insulin on neutral amino acid transport, ornithine decarboxylase activity, and the rate of growth of a kidney epithelial cell line (MDCK), In Vitro. *Cell. Dev. Biol. (submitted).*

lead to the suggestion that protein kinase C, at least in part, mediates the transcriptional regulation of system A in response to (a) TPA, (b) growth state, and (c) transformation.

D. Nutrient Transport and Regulation of Cellular Growth

The identification of the functionally relevant targets of oncogene- and protooncogene-encoded protein kinases is currently one of the most pressing goals of cancer research (Ratner, Josephs & Wong-Staal, 1985; Denhardt, Edwards & Parfett, 1986; Alitalo et al., 1987). Protein kinases participate in the regulation of cellular growth and differentiation as well as the regulation of transport systems which allow entry of essential nutrients into the cell cytoplasm (Boyer & Krebs, 1986; Edelman, Blumenthal & Krebs, 1987). In this section we shall summarize studies conducted primarily with the MDCK cell line (McRoberts et al., 1981) in which the coordinate regulation of system A with growth has been demonstrated. These cells have been examined both *in vitro* (in the tissue culture environment) and *in vivo* (in the athymic nude mouse) in order to establish the relevance of *in vitro* studies to the *in vivo* environment (Saier, 1981; Taub et al., 1981; Saier et al., 1982).

Growth regulation will first be discussed in general terms, and the nude mouse will be considered as an experimental system for the determination of the oncogenic potential of a cell line. The growth and differentiated properties of the MDCK cells will then be described, both *in vivo* and *in vitro*. We shall see that the growth of these cells is regulated by the two factors, insulin or an insulin-like growth factor and a prostaglandin of the E class (PGE_1 or E_2). Both factors appear to exert their effects by protein kinase-mediated mechanisms, although this proposal is by no means established. Subsequently, the characteristics of transformed variants of the MDCK cells will be considered. These transformed variants will be used to focus on the system A amino acid transporter which, in the MDCK cells as well as in several other cell systems, may be causally related to growth rate. The evidence that this system is a functionally relevant target of oncogene- and protooncogene-encoded protein kinases will be summarized, and the possible regulatory mechanisms controlling the synthesis and activity of the system will be discussed. Conceptualization of the regulatory phenomena outlined in this section provides a basis for understanding the step-by-step pathways by which protooncogenes control the biochemical systems which regulate growth in normal cells. In the last two sections, other transport systems such as the NaCl/KCl symporter, the Na^+/H^+ antiporter, the Na^+,K^+-ATPase and the glucose carrier, which may prove to be targets of oncogene action in various cell lines, will be briefly discussed. An understanding of the mechanisms by which overexpressed or altered protooncogenes disrupt normal regulatory controls follows as a logical extension of these considerations.

1. Regulation of Cell Growth: General Considerations

Recent investigations have revealed many details concerning growth regulatory mechanisms in tissue cells. Growth factors and their receptors, some which exhibit tissue specificity, have been identified and characterized, and a number of cytoplasmic "second messengers" (cAMP, cGMP, Ca^{2+}) have been identified (Heldin & Westermark, 1984). Effects of cellular protein kinases on growth have become established, and many of these kinases as well as the phosphatases which reverse their action have been characterized (Boyer & Krebs, 1986; Edelman et al., 1987). Additionally, several oncogenes and their protein products are known. These oncogenes code for growth factors and their membrane-bound receptors, protein kinases, and other regulatory proteins, such as the GTP-binding proteins which mediate the actions of various receptors (*see* Table 1 in Alitalo et al., 1987, for a compilation of functionally identified retroviral oncogenes). Corresponding chromosomal protooncogenes code for cellular proteins with normal functions involved in growth regulation. The products of the various oncogenes and protooncogenes form a molecular cascade which exerts its effect on growth and probably on cellular differentiation as well.

The products of other oncogenes (i.e. *fos* and *myc*) are probably among the substrates of the oncogene-encoded protein kinases (Ratner et al., 1985; Denhardt et al., 1986; Alitalo et al., 1987). But the functions of the *fos* and *myc* gene products are not known. In fact, none of the functionally relevant targets of oncogene-encoded protein kinases have been identified. The identification of these proteins is one of the most important goals of current-day cancer research.

Cancer cells are believed to arise from any of a variety of differentiated somatic cells. The fact that more than 80% of human tumors are carcinomas, and therefore arose from epithelial cells, provided the incentive for studies with this cell type. In order to allow use of the genetic approach, it was decided that a well-characterized cell line could be used with maximal benefit. We postulated more than a decade ago that transformation of a normal cell to

the cancerous state required two events; loss of cellular mortality and loss of sensitivity to host-generated growth regulatory stimuli (Stiles et al., 1976*a*,*b*, 1977). In order to study the latter process, an appropriate host animal was required in which tumor cells, but not normal cells, would proliferate. The athymic nude mouse proved to be the most acceptable animal of choice (Stiles et al., 1976*a*). Because this mutant mouse strain possesses only a rudimentary thymus, it is almost completely lacking in the cellular immune system (Rygaard, 1973). Because of this deficiency, these mice lack the ability to reject skin transplants or injected cells derived from any of a variety of sources. For example, embryonic chicken skin has been grafted onto the backs of nude mice, and the transplants not only grew but developed feathers. The nude mouse therefore appeared to be ideally suited for investigations of the factors and conditions controlling growth and expression of the differentiated state of any of a variety of animal cells.

In a series of investigations with many cell lines, Stiles et al. (1976*a*) established the suitability of the nude mouse for estimation of the tumorigenic potential of a cell line. Subcutaneous injections of any of a variety of cell lines derived from tumor tissue of either a human or an animal source invariably gave rise to cancerous growth in the nude mouse. Interestingly, these tumors never metastasized, regardless of their state of malignancy in the original host animal from which the oncogenic line had been isolated. This surprising and still unexplained observation has led to the postulate that metastasis in some unknown way is dependent on the host cellular immune system.

Cell lines that had been established in culture from normal cells without the use of viruses or chemical carcinogens were seldom tumorigenic in the nude mouse. On the other hand, cells established in culture from normal tissue, but with the help of viruses or carcinogens, frequently, but not always, generated tumors. These results led to the suggestion that the nude mouse is well suited for estimation of the oncogenic potential of a cell line.

The results of Stiles et al. (1976*a*), provided important evidence for the postulate that loss of cellular mortality and loss of sensitivity to growth-regulatory signals could occur as two distinct events. "Normal" cell lines such as the mouse embryo 3T3, dog kidney MDCK, rat liver BRL, and rat ovary 31-A lines, which are "established" but nontumorigenic, exemplified the stepwise process. On the other hand, the tumorigenic characteristics of lines established *in vitro* with the use of viruses or chemical carcinogens (i.e. the SV-40-transformed SVT2 and CD-1 lines, the polyoma-transformed PY3T3 line, and the methylcholanthrene-induced A-9 line) suggested that a single genetic alteration might simultaneously result in loss of both the program of mortality and sensitivity to host-generated growth regulatory signals. The presence of a "master switch" controlling both functions was implied.

Further work demonstrated that the *in vitro* tests then available for transformation did not serve as reliable indicators of oncogenicity (Stiles et al., 1976*b*). These assays included growth in media containing a low concentration of serum, growth to high cell density, growth in the absence of anchorage (i.e. in the absence of a solid support), and growth on a monolayer of normal mouse fibroblasts (the phenomenon of contact inhibition of growth). The results of these assays did not correlate with tumorigenicity *in vivo*. It was therefore concluded that of the methods available, tumor formation in the nude mouse provides the most reliable method for establishing the tumorigenic potential of a cell line from any mammalian source.

2. Growth and Differentiated Characteristics of MDCK Cells In Vivo

When MDCK cells were injected into adult nude mice, tumor formation was never observed. Control experiments established that these nongrowing cells were not killed or otherwise rejected. They could be recovered from the nude mice three months post injection (Stiles et al., 1976*a*). When the same cells were injected subcutaneously into baby nude mice, nodule formation was consistently observed. Moreover, when the host mouse reached adulthood, the nodules ceased growing, retaining the size attained when adulthood was reached. Regression was never observed (Stiles et al., 1976*a*, 1977).

Histological studies of the nodular tissue which resulted following injection of MDCK cells in the newborn animals (Rindler et al., 1979) revealed that the MDCK cells, together with mouse fibroblasts, had formed kidney-like tissue. The injected epithelial cells were found to border kidney tubule-like structures exhibiting all of the characteristics of distal tubular epithelia (Rindler et al., 1979). Sparse microvilli on the surfaces of apical membranes faced the lumen of fluid-filled sacs while smooth-surfaced basolateral membranes faced the basement membranous structure which separated the epithelioid from the fibroblastic elements (Rindler et al., 1979). The basement membranes were presumably synthesized by the combined action of the mouse fibroblasts and the dog epithelial cells. The cells forming the tubular structures were connected by tight and gap junctions as ob-

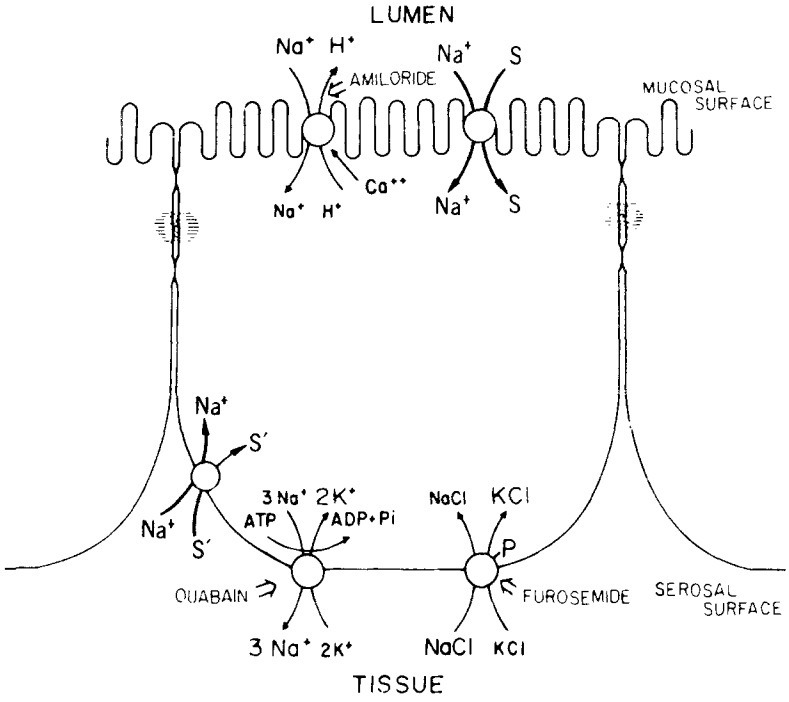

Fig. 2. Schematic depiction of an MDCK cell revealing the different cellular locations of various transport systems. These include the amiloride-sensitive Na^+/H^- antiporter and the Na^+-amino acid system G cotransporter in the apical (mucosal) membrane, and the ouabain-sensitive Na^+,K^+-ATPase, the furosemide-sensitive NaCl/KCl symporter and the system A amino acid transporter in the basolateral (serosal) membrane

served in intact kidney tissue, and kidney-specific multivesicular bodies were found. The tissue formed by this mixed population of cells was morphologically indistinguishable from normal kidney tissue (Rindler et al., 1979). Evidently, the MDCK cells have not lost the differentiated characteristics of their tissue of origin, even though they were isolated 30 years ago.

3. Growth and Differentiated Characteristics of MDCK Cells In Vitro

As first reported by Leighton et al. (1969), confluent monolayers of MDCK cells appear to transport salt and fluid in a vectorial fashion from the apical (mucosal, upper) surface of the monolayer to the basolateral (serosal, lower) layer of the monolayer growing on a plastic dish (Misfeldt et al., 1976; Cereijido et al., 1978*a*,*b*). This vectorial transport of fluid is believed to create sufficient hydrostatic pressure to dislodge clusters of cells from the surface of the plastic, causing them to be raised from the surface as blisters or domes (Cereijido et al., 1978*b*). The transport of salt and water occurs in the same direction (apical to basolateral) as in intact kidney tissue where transport results in retention of electrolytes and fluids as well as concentration of the urine. These observations as well as *in vitro* morphological and electrophysiological studies (Misfeldt et al., 1976; Cereijido et al., 1978*a*,*b*) confirmed and extended the *in vivo* work suggesting that MDCK cells have retained the functional characteristics of distal tubular kidney epithelial cells (*see also* Herzlinger, Easton & Ojakian, 1982).

Further physiological and pharmacological studies established that the vectorial salt and fluid transport in MDCK monolayers was due to the action of an amiloride-sensitive, Ca^{2+}-activated Na^+/H^+ antiporter present in the apical membranes (Lever, 1979; Rindler et al., 1979; Taub & Saier, 1979; Rindler & Saier, 1981) and the ouabain-sensitive Na^+,K^+-ATPase present in the basolateral membrane (Sharkey, 1983). The furosemide-sensitive, ATP-activated NaCl/KCl symporter present in the basolateral membrane was not involved (Fig. 2). The polarity of the MDCK cell surface is well established (Richardson & Simmons, 1979; Ü et al., 1979, 1980; Imhof et al., 1983; Misek et al., 1984; Simmons & Fuller, 1985; Boerner et al., 1986).

In order to determine the growth factor requirements for the MDCK cells, a serum-free, hormonally defined medium was developed (Taub et al., 1979). As shown in Table 2, the MDCK cells, like most other animal cells, require transferrin, insulin and selenium for growth *in vitro*. While transferrin provides the necessary iron, insulin, which must be supplied in superphysiological concentrations, presumably substitutes for the insulin-like growth factor type I or II (Blundell, Bedarkar & Humbel, 1983), probably type I (Kiess et al., 1987). In addition, two hormones which act in the nucleus, hydro-

Table 2. Quantitative requirements for continuous growth of the MDCK cell line in the absence of serum[a]

Transferrin	5 μg/ml
Insulin	5 μg/ml
Selenium	5×10^{-8} M
Hydrocortisone	5×10^{-8} M
Triiodothyronine	10^{-9} M
Prostaglandin E_1	25 μg/ml

[a] The growth rate in serum-containing medium was the same as in this defined medium suggesting that no other factors were required for optimal growth (Taub et al., 1981).

cortisone and triiodothyronine, were essential for maximal growth rates. Finally, an adenylate cyclase-stimulating hormone had to be supplied. While any of four different agents could be used [a prostaglandin of the E class, glucagon, vasopressin, or a catecholamine (in order of effectiveness)] a correlation was observed between long-term growth (Taub et al., 1979) and degree of adenylate cyclase stimulation (Rindler et al., 1979). The growth requirements of primary cultures of distal tubular epithelial cells from a variety of mammalian species were quantitatively the same as those of the MDCK cells (Taub et al., 1981; Taub, 1984*a*,*b*). This fact indicated that, in agreement with the *in vivo* studies in the nude mouse, the MDCK cells, while established in culture, have retained the growth and differentiated properties of the parental cell of origin, and that the characteristic of immortality is therefore genetically distinct from that of transformation.

4. Regulation of System A Synthesis by Insulin and Prostaglandin E_1 in MDCK Cells

Both insulin and prostaglandin E_1 have been reported to be essential for the adaptive response in MDCK cells (Boerner & Saier, 1985*b*). However, they appeared to exert their effects in different ways. While insulin removal prevented derepression, resulting in low basal activity which could not be enhanced in response to amino acid starvation, prostaglandin E_1 removal led to full derepression even in the presence of concentrations of exogenous amino acids which normally fully repress system A synthesis. The low activity of insulin-deprived cells correlated with a depressed growth rate, while the high activity of prostaglandin E_1-deprived cells correlated with an enhanced growth rate (Boerner & Saier, 1982*a*).

MDCK cells grow slowly (generation time of 36 hr) in defined medium supplemented only with selenium and transferrin.[3] That is, they are competent to undergo cell division, but their progression through the cell cycle is slow. Addition of insulin stimulates growth (generation time of 25 hr) and stimulates amino acid uptake via both systems A and ASC. The system A-specific substrate, α(methylamino)isobutyrate (MeAIB) was found to largely prevent the insulin-promoted enhancement of system A activity, and it partially inhibited growth. The stimulation of system ASC activity by insulin was not appreciably altered. These results are consistent with the suggestion that insulin stimulates progression through the cell cycle in part by enhancing the availability of cytoplasmic amino acids. System A and ASC therefore appear to be physiologically relevant targets of insulin action, and these two carriers act in concert to provide neutral amino acids for protein synthesis. Just as insulin stimulates tyrosyl protein kinase activity by binding either to its own receptor at physiological concentrations or to the insulin-like growth factor type I receptor at superphysiological concentrations of insulin (Boyer & Krebs, 1986; Edelman et al., 1987; Kiess et al., 1987), prostaglandin E_1 is believed to exert most if not all of its effects on MDCK cells by stimulating adenylate cyclase, enhancing cytoplasmic cyclic AMP concentrations, and thereby activating protein kinase A (Rinder et al., 1979; Boyer & Krebs, 1986).

These considerations lead to the suggestion that insulin promotes phosphorylation of a target protein which in the phosphorylated form functions as a positive effector of transcription to allow derepression of system A, while prostaglandin E_1 promotes phosphorylation of a different target protein which together with a repressive amino acid functions as a negative effector of system A transcription. It is possible that these two phosphorylation events interconvert the activator and repressor forms of a single transcriptional regulatory protein as has been demonstrated in bacteria (Deutscher & Saier, 1988).

5. Properties of Virally and Chemically Transformed Variants of the MDCK Cells

Transformed variants of the MDCK cells have been isolated by four procedures: 1) by selection for rapid *in vitro* growth in the absence of anchorage after mutagenesis with nitrosoguanidine or ethylmethane-sulfonate (Ü et al., 1985); 2) by selection for tumorigenicity in the nude mouse after chemical mutagenesis (Ü et al., 1985); 3) by selection for continuous growth of MDCK cells in hormonally defined medium lacking prostaglandin E_1 *in vitro* (Taub et al., 1983; Taub, Devis & Grohol, 1984); and 4) by selection for tumorigenicity after expo-

sure to Moloney sarcoma virus (MSV) (Taub et al., 1981; Ü et al., 1985). Each transformed cell type was clonally isolated and characterized with respect to its *in vivo* and *in vitro* growth properties. Chemically transformed MDCK cells, selected for anchorage independence *in vitro*, retained their epithelial morphology and were not tumorigenic in adult nude mice after 6–9 months *in vivo*. Chemically transformed MDCK cells, selected for tumorigenicity by growth in the nude mouse, gave rise to tumors six weeks postinjection (Table 3). Although they resembled the parental cell line with respect to serum requirement and absolute growth rate, they showed no requirement for prostaglandin for maintenance of viability, and they exhibited a diminished requirement for insulin (Ü et al., 1985). They synthesized increased amounts of cyclic AMP under both basal steady-state and hormone-stimulated conditions. These cells retained an epithelioid morphology but assumed a slightly elongated shape. Prostaglandin E_1-independent mutants were isolated after chemical mutagenesis (Taub et al., 1983, 1984). These cells were found to grow optimally in the MDCK hormonally defined medium lacking prostaglandin. In fact, prostaglandin E_1 or dibutyryl cyclic AMP plus isobutylmethylxanthine was markedly growth inhibitory to some of the mutants. Most prostaglandin-independent mutants possessed enhanced basal and hormone-stimulated levels of cyclic AMP. In contrast to the parental line, some of these mutant cell lines formed nodules in adult nude mice several months after subcutaneous injection (M. Taub, L.M. Chuman & M.H. Saier, Jr., *unpublished results*). MSV-transformed MDCK cells gave rise to tumors in nude mice two weeks postinjection. They showed serum requirements similar to those of the MDCK cells but possessed a fibroblastic morphology. Although these cells required the five growth factors of the MDCK-defined medium, a lower insulin concentration allowed maximal growth (Taub et al., 1981; Ü et al., 1985).

These results, taken together, implicate both prostaglandin E_1 (or another cyclic AMP stimulatory agent) and insulin (or an insulin-like, growth-promoting agent) in the regulation of kidney epithelial cells *in vivo* as well as *in vitro*. They further suggest that, at least in these cells, a depressed requirement for either of these growth factors can give rise to the neoplastic state. It is worth noting at this point that the two growth factors implicated in growth regulation *in vitro*, insulin and prostaglandin E_1, are the same factors that influence amino acid uptake via the A-transport system *in vitro* (*see* section C5). Because the chemically transformed line, MDCK-T_1, most resembled the parental MDCK line, with the exception of its tumorigenic characteristics, MDCK-T_1 cells were extensively characterized.

Table 3. Properties of the tumorigenic MDCK-T_1 cells

1. Tumorigenic in adult athymic nude mouse.
2. Loss of growth dependencies on prostaglandin and insulin.
3. Elevation of cytoplasmic cyclic AMP levels.
4. Normal responses of adenylate cyclase to stimulatory hormones.
5. Normal activities of the Na^+,K^+-ATPase, the Na^+/H^+ antiporter, and the NaCl/KCl symporter.
6. Normal rates of glucose uptake.
7. Derepression of System A amino acid transport, but other amino acid transport systems appear normal.

6. Comparison of Normal and Transformed MDCK Cells: Growth, Protein Content, Cell Volumes and Transport Characteristics

The growth of MDCK and MDCK-T_1 cells in serum-free medium was quantitated and compared. Both cell lines grew exponentially with the same generation time after similar lag phases (Erlinger & Saier, 1982). Growth rates in serum-containing medium were also similar.

Protein content of the two cultures was studied as a function of cell number during growth. Protein content increased with cell number until the cells approached confluence. Thereafter, cell number increased without any further increase in protein content. The protein content per cell showed a sevenfold decrease as cells went from the sparse to the highly confluent state. No difference was detected between MDCK and MDCK-T_1 cells or between cells grown in serum-containing or serum-free medium. Cell volume decreased in parallel with cell protein.

An attempt was made to assess the influence of the reference (cell number, cell protein or cell volume) on the results of transport measurements with MDCK and MDCK-T_1 cells during growth at increasing cell densities (Erlinger & Saier, 1982). When expressed per 10^6 cells, $^{22}Na^+$ uptake in the absence of K^+ was markedly higher in sparse than in subconfluent cells. In contrast, when expressed per mg protein, $^{22}Na^+$ uptake was almost identical at the two cell densities, whereas it was slightly higher in subconfluent than in sparse cells when expressed per μl of cell water. As for the K^+-stimulated rate of Na^+ uptake, the K^+-independent Na^+ uptake rate was the same in the MDCK and MDCK-T_1 cells. It was therefore concluded that neither growth state nor transformation appreciably

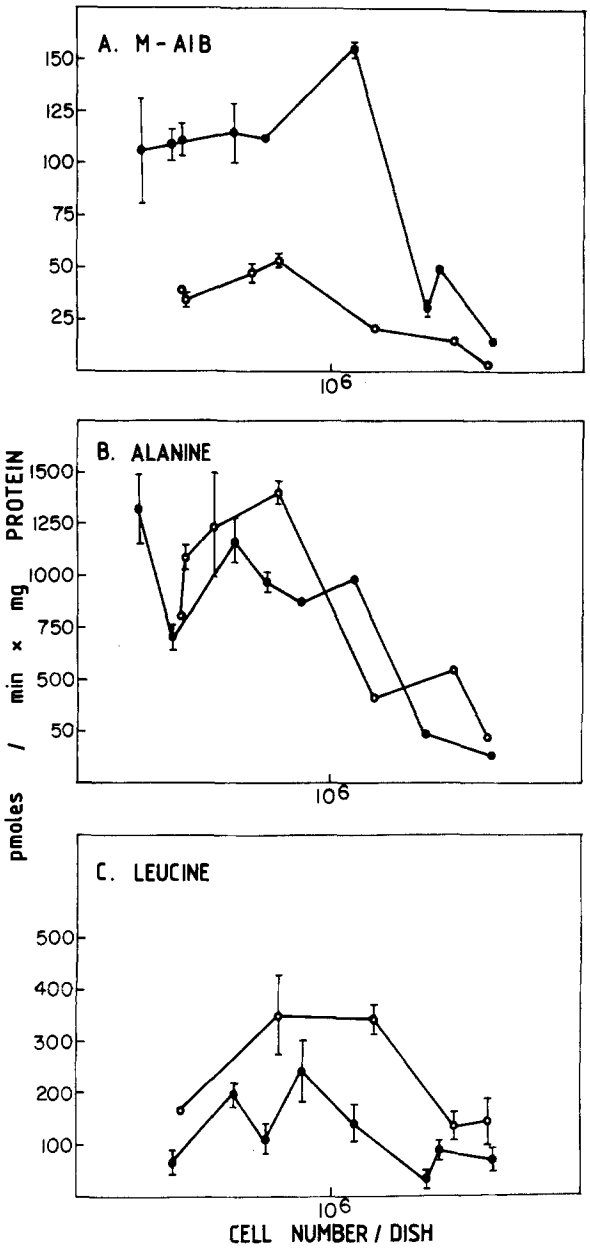

Fig. 3. Effects of cell density and transformation of the activities of the A (*A*), ASC (*B*) and L (*C*) amino acid transport systems in MDCK (○) and MDCK-T_1 (●) cells. (Reproduced from Boerner and Saier, 1982*a*, with permission)

altered either the NaCl/KCl symporter or the Na^+/H^+ antiporter (*see*, however, Reznik, Villela & Mendoza, 1983).

Glucose uptake was also measured as a function of cell number in the MDCK and MDCK-T_1 cells. When expressed per 10^6 cells, there was a threefold decrease in uptake from sparse to confluent cells, but when expressed per μl of cell volume or mg protein, no significant change was noted. No differences were observed between the MDCK and MDCK-T_1 cells under the conditions employed (Erlinger & Saier, 1982).

7. System A as a Possible Determinant of Growth Rate in MDCK Cells

A single epithelial cell line had not previously been the focus of a comparative study of the responses of amino acid transport systems to different growth conditions. The MDCK cell line was deemed well suited to studies of amino acid transport regulation as they relate to growth regulation and transformation, first, because the use of a fully defined medium allowed the effects of growth factors on transport activity to be examined in detail, and second, because the availability of the MDCK-T_1 line allowed differences in transport activity to be attributed to transformation rather than to changes in growth rate.

The Na^+-dependent systems A and ASC and the Na^+-independent system L (Oxender & Christensen, 1963; Christensen et al., 1967; Kilberg et al., 1981; Colarini & Oxender, 1987) were compared when growth states were altered by manipulating the cell density, hormonal composition of the medium, and the oncogenic state of the cell (Boerner & Saier, 1982*a*,*b*; *see* Fig. 3). The results obtained showed that Na^+-independent transport of leucine or methionine (via system L) responded minimally to environmental manipulation. The activities of both Na^+-dependent transport systems, however, decreased severalfold in response to increasing cell density, and both systems, in nonconfluent, low density cells, required insulin for optimal activity. In contrast, system A responded differently from system ASC with respect to regulation by prostaglandin E_1 and transformation. The activity of system A was three- to sevenfold higher in the MDCK-T_1 cells than in the parent at every stage of growth, whereas the activity of system ASC was similar in the two cell types (Fig. 3). Removal of prostaglandin E_1 from the growth medium of low density MDCK cells for 24 hr resulted in a 90% increase in system A transport activity, whereas system ASC transport activity was unchanged (Boerner & Saier, 1982*a*).

Further studies on system A regulation revealed that the phenomenon of adaptive regulation (*see* section C.2) only occurred when the MDCK cells were grown in the presence of insulin and prostaglandin E_1 (Boerner & Saier, 1985*a*,*b*). Thus, as noted in section D.4, low activity which could

not be derepressed was observed in the absence of insulin, but high activity, equivalent to the fully derepressed state, was chronically observed in the absence of prostaglandin E_1. When both factors were omitted, intermediate activity was observed (Boerner & Saier, 1985*b*). It appeared that insulin acted as a positively controlling element while prostaglandin E_1 acted as a negative controlling factor, and that the two factors acted independently of one another. As noted previously, these results correlated with growth. Surprisingly, in the MDCK-T_1 cells, in which growth is independent of both agents and cyclic AMP levels are elevated, system A is chronically derepressed as in MDCK cells when grown with insulin but without prostaglandin E_1.

Kinetic analyses of system A activity in MDCK and MDCK-T_1 cells after growth under a variety of conditions revealed that while repressed MDCK cells exhibited linear kinetics with a low apparent affinity (high K_m value), derepressed MDCK cells or MDCK-T_1 cells grown under any of a variety of conditions exhibited biphasic kinetics with one of the two components exhibiting high substrate affinity (a low K_m value). The data were consistent with the conclusion that transformation resulted in (a) high level constitutive expression of system A and (b) the appearance of a second high affinity form of the system A carrier.

More recently it has been found that derepression in the presence of insulin, PGE_1 and saturating concentrations of repressive amino acids can be elicited in MDCK cells (but not MDCK-T_1 cells) by exposure for several hours to phorbol esters (TPA).[1] Since the elevation of system A activity elicited by PGE_1 removal, amino acid starvation, phorbol ester addition, or transformation could be blocked by addition of a saturating concentration of sodium butyrate, a single regulatory mechanism was postulated. Since butyrate is known to prevent histone deacylation, it is possible that a deacylation reaction is responsible for system A derepression. Further, since phorbol esters are believed to exert their effects on protein kinase C (Dawson & Cook, 1987; *see* section C.5) it can be suggested that protein kinase C regulates system A synthesis by phosphorylating a protein which in the phosphorylated state either inhibits the relevant acylation reaction or stimulates deacylation. Such speculations are of little further value until the genetic apparatus controlling system A synthesis is available for study. It is interesting to note that azacytidine, an inhibitor of DNA methylation, inhibited basal (amino acid repressed) system A activity but did not block the increase in activity which resulted upon amino acid starvation.[1] It can therefore be suggested that the DNA methylation state controls the potential for basal system A expression but not the responsiveness of its expression to physiological conditions.

The genetic approach has recently been applied to this problem.[2] In experiments designed to establish the relationship of system A to oncogenicity, MDCK-T_1 cells, which were found to be more sensitive than MDCK cells to inhibition of growth by MeAIB, were mutagenized, and MeAIB-resistant variants were isolated. These variants exhibited reduced system A activity which was intermediate between that of MDCK and MDCK-T_1 cells. Several of these clones were investigated further with respect to (1) MeAIB transport kinetics, (2) adaptive regulation of system A activity, (3) ornithine decarboxylase activity, and (4) oncogenicity in the nude mouse. The sensitivity of the A-transporter to repression by amino acids, the absolute V_{max} values for system A transport, and the levels of ornithine decarboxylase activity were not found to correlate with oncogenicity. However, oncogenicity did show a correlation with the presence of the higher affinity form of the A-transporter. It was therefore postulated that (a) hormonal agents (insulin and PGE_1), (b) repressive amino acids, and (c) oncogenic potential might influence growth *in vivo* as well as *in vitro* by controlling the expression of the high affinity form of the A-transporter. It was further suggested that the presence of the high affinity transporter might provide a reliable *in vitro* assay for the tumorigenicity of certain cell types.[2] It should be noted that some of the mutations altered system ASC as well as system A, and that the combined action of the A and ASC systems rather than system A alone may be the physiologically significant parameter controlling growth.

8. Evidence for the Common Regulation of Growth and the NaCl/KCl Symporter by a Protein Kinase

The NaCl/KCl symporter transports four ions: 1 Na^+, 1 K^+ and 2 Cl^- in the same direction in a reversible, energy-independent process which allows these ions to equilibrate across the membrane in accord with their chemical potentials (Saier & Boyden, 1984; *see* Fig. 2). Studies with energy poisons led to the conclusion that the system is activated by ATP, probably by a protein kinase-catalyzed process (McRoberts et al., 1982; Rindler, McRoberts & Saier, 1982). Mutants lacking the activity of the system were isolated and found to fall into two classes: One class, represented by the mu-

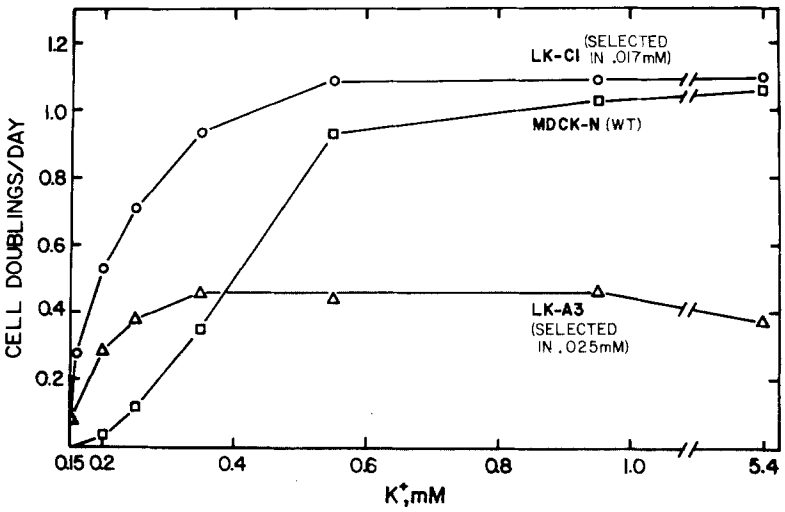

Fig. 4. Growth of MDCK cells and two mutants selected on the basis of enhanced growth rates in medium containing low potassium (<0.2 mM). Growth rate is plotted as a function of the K^+ concentration in the external medium. The two mutants shown are LK-C1 and LK-A3, both of which lack demonstrable NaCl/KCl symport activity. (Reproduced with modification from McRoberts et al., 1983)

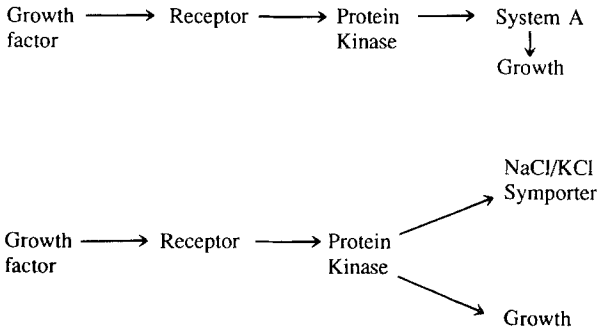

Fig. 5. Proposed, simplifed pathways for the simultaneous control of growth with that of two transporters in the MDCK cells: the system A carrier, and the NaCl/KCl symporter

tant LK-Cl, grew in medium containing high K^+ concentrations at the same rate as did the parental MDCK cells (Fig. 4). The second class, represented by mutant LK-A3 in Fig. 4, grew optimally at a rate which was only 30% as fast as the parental MDCK cells. Since both mutant classes totally lacked NaCl/KCl symport activity, the transport system was obviously not required for growth. This conclusion was in agreement with the previous studies summarized in section D.6. Based on these properties it can be postulated that the first mutant class with normal growth at high K^+ is defective for the transport protein itself, while the second mutant class with a reduced growth capacity at high K^+ is defective for a protein kinase which positively regulates growth and also activates the symporter. This suggestion is in agreement with the conclusion, noted above, that NaCl/KCl symport activity is dependent on ATP and a protein kinase-catalyzed phosphorylation event. It appears to be substantiated by a recent report (Giesen-Crouse & McRoberts, 1987) showing that while the symporter could not be detected by piretanide binding in the LK-Cl cells, low affinity binding of this loop diuretic was demonstrable in the LK-A3 cells.

9. Transport Systems as Targets of Oncogene Action

Many well-characterized growth factors interact with their membrane receptors to activate protein kinases (Fig. 5). One such protein kinase in MDCK cells presumably regulates synthesis of the A-amino acid transporter which in turn controls growth (Fig. 5, top). A second protein kinase which is postulated to be activated by a different factor and its receptor activates the NaCl/KCl symporter and independently stimulates cell growth (Fig. 5, bottom). In the latter but not the former case, the coordinate control of transport and growth is thought to reflect the fact that a single protein kinase independently regulates several distinct enzyme systems by phosphorylating several target proteins. Still another carrier believed to be regulated by phosphorylation is the system G amino acid transporter, found in the apical membrane of MDCK cells (Boerner et al., 1986). Since this system is activated by exogenous dibutyryl cyclic AMP as well as adenylate cyclase-stimulating hormones, protein kinase A may be involved (Boerner et al., 1986; D. Dean, *unpublished*).

Numerous transport systems are now believed to be regulated by protein kinases. The best characterized systems which are regulated by protein kinases and which are also believed to influence growth rate are presented in Table 4. Many of these serve as potential determinants of growth rate and targets on oncogene action (Russel, Byus & Manen,

Table 4. Transport systems likely to be causally related to transformation and cellular growth state

Transporter	Cell System	Effect	References
System A	MDCK; LLC-PK$_1$; fibroblasts; CHO cells	Synthesis and activity stimulated by insulin, TPA and a protein kinase C-dependent mechanism; also responsive to growth state and cyclic AMP-stimulating hormones.	this review
Glucose carrier	fibroblasts; adipocytes	Activity, synthesis and cellular localization influenced by TPA, protein kinase C and insulin	Carruthers, 1984; Christopher, 1977; Garry et al., 1986; Gibbs et al., 1986; Hyslop et al., 1985; 1987; Lee and Lipmann, 1977; Oka and Czeck, 1984; Salter et al., 1982; Weber et al., 1984; White et al., 1981.
Na^+/H^+ antiporter	fibroblasts; epithelial cells; neuronal cells; hepatocytes; lymphocytes; myoblasts;	Activity regulated by internal [Ca^{2+}], but probably not by phosphorylation. Transformation and growth state influence activity in some, but not other cells.	Koch and Leffert, 1979; Lagarde et al., 1988; Leffert and Koch, 1985; Paris and Pouyssegur, 1984; Reznik et al., 1983; Rindler et al., 1979; Rosengurt, 1980; 1986; Rothenberg et al., 1983; Skaper and Varon, 1987; Vigne et al., 1984.
Na,K^+-ATPase	fibroblasts; Friend leukemic cells	Activity possibly inhibited by a protein kinase and responsive to growth state.	Johnson and Weber, 1979; 1980; Skaper et al., 1986; Ulug et al., 1984; Varon and Skaper, 1983; Weber et al., 1984; Yeh et al., 1983.

1976; Bhargava, 1977; Rozengurt, 1980, 1986). Indeed, extensive evidence suggests that the Na^+/H^+ antiporter as well as the Na^+,K^+-ATPase are primary determinants of growth rate in many cell lines, while the glucose carrier may be a principal target of oncogene action in many others (*see* Table 4).

The biochemical nature of many oncogenes has now been elucidated (Ratner et al., 1985; Alitalo et al., 1987). However, dozens of oncogenes remain to be identified functionally. Many of these (such as *fos* and *myc*) will undoubtedly prove to be the targets of other oncogenes such as those encoding protein kinases (Alitalo et al., 1987). While the ultimate targets of oncogene action have in no instance been identified, we postulate that many such targets will prove to be transport systems and the genetic apparatuses controlling their syntheses. Molecular genetic and biochemical approaches are likely to prove most useful in establishing this hypothesis.

E. Summary and Perspectives for Future Research

In this review we have attempted to evaluate the published literature concerned with the regulation of neutral amino acid transport and its relationship to growth regulation in selected, well-characterized tissue cells. Although a number of these transport systems have been identified and characterized, (section B) the activity of only one, the system A carrier, (section C) has been shown to correlate consistently with growth potential in certain cell lines (section D). System A, together with the glucose carrier, the Na^+/H^+ antiporter, and the Na^+,K^+-ATPase, represents a potential determinant of growth rate in a tissue cell. The response of system A to environmental stimuli, growth factors and oncogenic transformation, and the apparent dependency of its synthesis on protein kinase C all support the contention that system A is a primary

target of oncogene action and a crucial regulator of cellular growth.

Because of the relationship of system A regulation to growth regulation, current research in several laboratories is focusing on the pathways and mechanisms by which the system A carrier is regulated. These studies will require that the cell surface receptors which regulate growth and transport be characterized, that the protein kinases which are responsive to growth factor stimulation and which control transport function be identified, and that the genetic apparatus encoding the system A carrier and its auxiliary regulatory constituents be cloned, sequenced, and analyzed. Cloning of these genes will permit detailed molecular genetic analyses of the transcriptional regulatory mechanisms responsible for the control of system A synthesis. Purification of the carrier protein should allow definition of the regulatory interactions which directly control its function. Any such studies will of necessity require the exploitation of a well-characterized cell system in which the effects of environmental stimuli, repressive amino acids, growth factors, growth state, and oncogenic transformation are all defined. The MDCK cell line or the CHO line appears to offer such a system. Further pursuit of the research problems discussed and evaluated in this review are likely to lead to a detailed understanding both of the mechanisms by which transport systems in normal cells respond to external stimuli and of the disruptive forces which give rise to the unregulated growth which characterizes the transformed state.

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