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CARRIER-MEDIATED MEMBRANE TRANSPORT OF FOLATES IN MAMMALIAN CELLS

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ABSTRACT

Mediated internalization of folates is required for cellular macromolecular biosynthesis. Multiple carrier-mediated mechanisms have been identified that can fulfill this role in a variety of mammalian cell types, including neoplastic cells, with and without proliferative potential. The absorption of dietary folates also relies on the function of a carrier-mediated system in mature luminal epithelium of small intestine. The various carrier-mediated systems can be distinguished by their preferences for various folate compounds as permeants as well as by differences in temperature and pH dependence. The widely studied one-carbon, reduced-folate transport system is mediated by a transporter encoded by the newly discovered RFC-1 (reduced-folate carrier) gene. The characteristics of this gene in rodent and human cells are similar, consistent with the close similarity between these species of folate transport mediated by this transporter. However, differences occur in the form of tissue-specific expression, alternate splicing, and 5′ end mRNA heterogeneity, as well as in promoter utilization regulating transcription. RFC-1 gene expression also appears to regulate luminal epithelial cell folate absorption in small intestine. However, the properties of RFC-1–mediated folate transport in these cells is anomalous when compared with that seen in nonabsorptive cell types. Detailed mechanisms as to the regulation of RFC-1 transcription are now

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emerging along with other information on structure and function of the transporter and its alteration following mutation.

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INTRODUCTION

Macromolecular biosynthesis is dependent on the intracellular availability of folate coenzymes that serve as cofactors (7, 59) for various folate-dependent enzymic reactions. Thus, tissues in the embryo and with proliferative capacity in the adult mammalian organism with a reliance on the biosynthesis of nucleic acid and proteins have strict requirements for folates. In view of the inability of mammalian cells to synthesize this vitamin de novo, these requirements must be satisfied from nutritional sources. Folates are highly lipophobic bivalent anions that can only minimally traverse biological membranes by simple diffusion (22, 38). Therefore, their internalization through mammalian cell plasma membranes must occur by means of a mediated process (22, 38, 80). Systems that translocate folates through biological membranes are vital at two levels within the intact mammal. They are necessary (*a*) for the absorption of folates, via the mature luminal epithelial cells of small intestine (91), and for reabsorption by a similar epithelial cell layer (65, 80, 84, 92) in proximal renal tubules and (*b*) for internalization through the plasma membrane of various proliferative and nonproliferative cellular elements within the intact embryo and adult organism.

These systems are also involved in the transmembrane movement of folates within the choroid plexus (17, 80, 85, 96, 106, 107), placenta (3, 80, 108), and various subcellular organelles (4–6, 51).

Two types of transport processes exist for the purpose of internalizing folates intracellularly. Carrier-mediated[1] processes (22, 38, 80) utilize binding to a transporter at the membrane surface to mediate internalization through membranes. Because of the mobile nature of the transporter (22, 38, 80), this process is capable of mediating bidirectional flux. Receptor-mediated processes (2, 58, 66, 80, 92) utilize high-affinity binding of folates at the membrane surface to a receptor-like protein and mediate unidirectional flux following internalization of the reception-folate complex in the manner (1, 104) of many other ligand-receptor processes. The extent to which carrier- or receptor-mediated processes contribute to net translocation of folates in those cell types where both processes are found is controversial (80), but it will depend on the level of expression of the corresponding gene in each cell type. Because the translocation efficiency of carrier-mediated processes is much greater (43, 58) than that of receptor-mediated processes, the relative level of expression required for the latter to contribute significantly to net translocation of folates is proportionally greater. Net intracellular accumulation of folates also reflects the operation of outwardly directed ATPases (41, 87, 89), which appear to be one or more members (60, 67) of the cMOAT/MRP family of ATPases found in the plasma membrane of a variety of mammalian cell types. ATPases within this group are also present within the membrane at the hepatocyte canicular surface and are involved (67) in biliary excretion of folates from the liver. From the above, it is evident that folate transport through biological membranes reflects the action of processes that are mechanistically diverse, fulfilling for mammals multiple functions that impact on folate homeostasis. Because of space constraints, this review focuses only on carrier-mediated processes, as they are within the scope of our own investigative experience.

PROPERTIES, MULTIPLICITY, KINETICS, AND STRUCTURAL SPECIFICITY OF CARRIER-MEDIATED FOLATE TRANSPORT

Criteria for Carrier-Mediated Membrane Transport

Carrier-mediated systems transporting folates have a variety of properties in common. The internalization (influx) of folates by these systems is saturable,

[1]In this article, the terms *carrier* and *transporter* are used interchangeably depending on the context. We believe the term *transporter* is preferable in some contexts because of the greater descriptive nature of the term.

conforming to Michaelis-Menten kinetics (22, 38, 96). However, they exhibit (22, 38, 96) differences in preferences for structurally related folates and their analogues, which are competitive inhibitors. The most convincing evidence for a system utilizing a mobile carrier relies on experiments (36) showing that the system is capable of exchange diffusion (26), where transpositioning of folate at one side of the membrane will either stimulate or inhibit the translocation of folate from the opposite membrane surface (36). After the internalization of folate, egress (efflux) by the transporter from the opposite side of the membrane occurs with increasing velocity, and internalization proceeds to steady state (influx = efflux) (22) in an exponential manner. In the case of 4-aminofolate analogues, such as methotrexate, which nearly stoichiometrically bind to cytosolic dihydrofolate reductase, unidirectional flux into the cell can be demonstrated (22, 38, 96). For this reason, in order to carry out valid kinetic measurements of influx, these analogues are often used as model permeants.

Unfortunately, these criteria described above for carrier-mediated transport are not always evaluated for specific systems translocating folates. Workers sometimes rely on surrogate criteria, some of which is described below. Carrier-mediated folate transport will often differ in regard to pH optimum and temperature dependence for mediated flux. Low temperature dependence (Q_{10} 27°–37°C < 3) is generally characteristic (26, 61) of an equilibrating rather than concentrative process operating in the form of facilitated diffusion. In contrast, highly temperature-dependent folate transport (Q_{10} 27°–37°C > 3) reflects active transport in energetically replete cells (26, 61), and folates accumulate (22, 38, 96) as anions within a compartment against an electrochemical concentration gradient.

Folate Transport in Neoplastic Cells

THE ONE-CARBON, REDUCED-FOLATE ROUTE Folates and their analogues are internalized in neoplastic cells, primarily by the one-carbon, reduced-folate transport system, the system most widely studied among mammalian cells (22, 38, 80). In comparison to all other transport routes identified in these cells, among rodent and human neoplastic cell types, the basic kinetic properties and preferences among structurally related folates and their analogues as permeants for the one-carbon, reduced-folate system are remarkably similar (Table 1). Most studies of this transport route have been carried out with murine L1210 cells. However, in almost all tumor cells, this route exhibits (Table 1) relatively low capacity but high saturability for mediated influx of both 5-substituted, reduced folates (K_m = 1–2 μM) and 4-aminofolate analogues (K_m = 0.5–10 μM) but extremely low saturability for folic acid (K_m = 50–200 μM). It is interesting that this transport route exhibits (97) stereospecificity at carbon 6, preferring the natural over the unnatural diastereoisomer of 5-formyltetrahydrofolate (47), but that it exhibits no stereospecificity for

Table 1 Summary of data derived on mediated entry of folate compounds in tumor cells

			Methotrexate influx		Influx K_m or K_i (μM)			
Species	Tumor	Type	V_{max} (nmol/min per m of g protein)	K_m or K_i (μM)	5ℓ L-Methyltetra-hydrofolate	5ℓ L-Formyltetra-hydrofolate	Folic acid	Reference
Mouse	L1210	Lymphoid leukemia	6.8 ± 1.9	3.9 ± 1	1.7 ± 0.2	2.2 ± 0.3	228 ± 35	37, 98
	P288	Lymphoid leukemia	2.5 ± 0.5	4.9 ± 1	0.9 ± 0.1	1.6 ± 0.2	188 ± 51	98
	P388	Lymphoid leukemia	2.6 ± 0.4	5.4 ± 1	1.3 ± 0.2	1.5 ± 4	176 ± 43	98
	L5178Y	Lymphoid leukemia	3.4 ± 0.6	3.0 ± 0.4	~2	~2	>200	40, 56
	Friend	Erythroleukemia	1.1 ± 0.4	3.6 ± 1	1.8 ± 0.6		>100	20
	M5076	Reticulosarcoma	1.2 ± 0.3	26.2 ± 6	1.6 ± 0.3	2.2 ± 0.3	>200	102
	Sarcoma 180	Sarcoma	5.9 ± 0.7	10.4 ± 2	1.0 ± 0.2	1.6 ± 0.3	216 ± 41	98
	Ehrlich	Carcinoma	2.8 ± 0.6	6.2 ± 1	1.5 ± 0.3	2.4 ± 0.3	253 ± 36	98
Rat	Yoshida	Sarcoma	2.1 ± 0.5	2.3 ± 0.4	~2	~2	>200	23
	H35	Hepatoma	1.1 ± 0.2	5.2 ± 1.4	<2		>100	30, 33
Hamster	CHO(Pro$^-$)	Ovarian carcinoma	1.0 ± 0.1	3.8 ± 0.1	1.0 ± 0.3		22	29
	DC3F	Lung carcinoma	3.4 ± 0.4	3.7 ± 0.5		1.9 ± 0.3	180 ± 26	96
Human	Manca	B-cell leukemia	2.5 ± 0.5	6.2 ± 0.5		1.2 ± 0.2	152 ± 30	71
	CEM-CCRF	Lymphoid leukemia	3.4 ± 0.6	5.9 ± 1.5	1.1 ± 0.3	2.2 ± 0.4	>100	57, 115
	W1-L2	Lymphoid leukemia	2.9 ± 0.4	6.7 ± 0.8	<2	4.2 ± 0.7		75
	HL60	Promyelocytic leukemia	1.3 ± 0.2	4.2 ± 1.1		1.3 ± 0.3	220 ± 35	101
	SKBRIII	Mammary carcinoma	1.2 ± 0.2	5.4 ± 0.6		1.4 ± 0.1	110 ± 10	71
	SK-Ov-1	Ovarian carcinoma	2.6 ± 0.4	3.9 ± 0.4		1.31 ± 0.3	150 ± 25	71
	MCF7	Mammary carcinoma	12.2 ± 2.8	8.2 ± 0.6		2.4 ± 0.4	>100	88
	K562	Erythroleukemia	4.86 ± 1	4.6 ± 1				68

5-methyltetrahydrofolate (97, 116). This transport route among different cell types (22, 38, 80, 96) shows a similarly high degree of temperature dependence (Q_{10} 27–37°C = 6–8, Arrhenius constant = 30–40 kcal) and a mild alkaline pH optimum as well as accelerated exchange diffusion. Other studies with L1210 cells revealed (14) that the capacity of one-carbon reduced-folate transport (influx V_{max}) was regulated in the form of growth-phase dependence. Cells in mid–logarithmic phase of growth had a threefold higher V_{max} for folate analogue influx compared with cells in stationary or early logarithmic phases. Following up on earlier studies by Goldman (37), Henderson & Zevely (44, 45) showed in L1210 cells that one-carbon, reduced-folate transport may act as an anion exchanger. That is, a variety of organic and inorganic anions will not only compete (44, 45) with folate compounds for mediated influx, they will also stimulate (44) efflux of internalized folate compounds. Kinetic analysis also showed (44) that both effects (inhibition and transstimulation) occurred at the same half maximal concentration, and the relative effectiveness among anions was the same in each case. On the basis of these results, it was proposed (37, 44) that the internalization of folates, which occurs against an electrochemical concentration gradient (uphill transport) (37), was a consequence of downhill counterflow of intracellular anions.

A reexamination of this anion-exchange model was carried out using L1210 cell plasma membrane vesicles (123), which essentially duplicated the effect of external anions on influx and efflux of a folate analogue. The results obtained also documented the stimulation by these anions within the intravesicular compartment on folate analogue influx (123), an effect that could not be demonstrated with intact cells. Moreover, it could also be shown that a gradient (internal/external) of either phosphate or sulfate stimulated influx and intravesicular concentrative accumulation of folate analogue from the external compartment. Thus, for the first time the downhill flow of anions within the internal compartment achieving concentrative internalization was directly demonstrated. However, the extent to which this could occur was unclear, because a positive gradient (internal/external) of total anions in the system was necessary to stimulate uphill counterflow of folate analogue. In addition, the gradient (internal/external) of either phosphate or sulfate required (123) to bring about significant uphill transport in the presence of a physiological level of chloride was 10-fold higher than that required under nonanionic buffer conditions. Thus, under these physiological conditions, the concentration of inorganic counter-anion necessary to stimulate influx was substantially higher than that normally found (44, 45) within the intracellular water of intact cells. It is entirely possible, however, that other anions, particularly organic phosphates as yet unidentified, with greater interactive potential for the carrier may operate in cells to bring about concentrative accumulation by this mechanism under physiological conditions.

OTHER CARRIER-MEDIATED ROUTES Although the one-carbon, reduced-folate transport route can internalize folic acid, albeit with much lower efficiency, evidence has been obtained for an additional route internalizing this folate in L1210 cells (122) that appears to contradict earlier results by others (55). Using plasma membrane vesicles, a transport route was identified under anionic buffer conditions that had relatively low affinity (influx $K_m = 400–500\ \mu M$) for folic acid but a maximum capacity (influx V_{max}) that was 20-fold higher than for the one-carbon, reduced-folate transport route. Moreover, compared with the one-carbon, reduced-folate route, this route had an even lower affinity ($K_m \geq 1000\ \mu M$) for folate coenzymes and 4-aminofolate analogues and a different sensitivity to the stilbene inhibitors 4,4′-diisothio-cyano-2,2′-disulfonic acid stilbene and 4-acetamido-4′-isothiocyano stilbene-2,2′ disulfonic acid. Because influx of folic acid by this route was not inhibited by adenine, an inhibitor of pteridine influx, it was unlikely that the compound actually being internalized was a pteridine decomposition product in the sample of [^{3}H] folic acid used as permeant, a complication suggested by others (55). Also, influx of folic acid by this route was unaltered in L1210 cell variants (122) with a defective one-carbon, reduced-folate transport system. Using other criteria (99), the existence of this low-affinity/high-capacity route for folic acid influx could not be demonstrated (99) in a nonanionic buffer with intact L1210 cells. However, the duality in routes for folic acid influx could be seen (99) when physiological chloride was added to the nonanionic buffer. Under monanionic buffer conditions, the documented (44, 123) interference of chloride with the interaction between folic acid and the one-carbon, reduced-folate transporter was eliminated. Also, folic acid influx mediated by the same transporter in cells suspended in nonanionic buffer was no longer enhanced by the downhill counterflow of anions (see above). Thus, these results appear to explain the discrepancy between studies by Huennekens et al (55) and that by Sirotnak and colleagues (99, 122).

Other earlier studies by Henderson et al (42) delineated in L1210 cells an acid pH-dependent route exhibiting similar affinity ($K_m = 4–6\ \mu M$) for folic acid, folate coenzymes, and folate analogues. The maximum capacity of this route was substantially less (42) than for the one-carbon, reduced-folate transport route and could only be detected in an L1210 cell variant in which this transport route was genetically compromised. Verification of the existence of this acid pH-dependent route in a similar L1210 cell variant was recently reported (94). However, its significance for the internalization of folates in parental L1210 cells is unknown.

Plasma Membrane Systems Identified in Nonabsorptive Normal Cells

FIBROBLASTS AND ERYTHROID CELLS Inward transport of folate compounds occurs in fibroblasts and erythroid cells by a system that is similar, if not

identical, to the one-carbon, reduced-folate system in tumor cells. This system in primary embryonic fibroblasts from mice and in immortalized murine and human fibroblasts exhibits (96) the same relative preferences among folate compounds. Influx V_{max} in the latter case was much higher, particularly following viral transformation (96). A similar system with the same preferences for folate compounds was also expressed (8–10) in rabbit and human erythrocytes. The influx V_{max} in these mature blood cells was two to three orders of magnitude lower than that of tumor cells but was increased (8–10) 15-fold following phenylhydrazine-induced reticulocytosis. The physiological significance of this system in erythrocytes is dubious given the extremely low levels at which it operates. Still, it was of interest to observe (8–10) that induction of proliferation in these cell types resulted in enhanced functional expression of folate transport.

HEPATOCYTES In rat hepatocytes, mediated influx of folates and their analogues showed both multiplicity and kinetic diversity (31–35, 49, 50). A system with high affinity for the folate analogue permeant (methotrexate) was found (31, 32, 35) that had an extremely low capacity (influx V_{max}) and was not competitively inhibited by natural folates, including 5-methyl and 5-formyl-tetrahydrofolate and folic acid. There also appeared to be a system with high affinity for 5-methyltetrahydrofolate that was competitively inhibited by methotrexate (50). These two high-affinity systems were further delineated by their different metabolic stability during primary cultivation of rat hepatocytes with various hormones (50). A third transport system exhibited low affinity for all folate compounds (49). It is interesting that in contrast to one-carbon, reduced-folate transport in tumor cells, all three of these transport systems in hepatocytes were found to be sodium dependent and quabain sensitive (31–35, 49, 50). Moreover, recent work by Horne (48) showed that the two high-affinity systems in rats exhibited a different pH dependence. Using isolated basolateral membrane vesicles, Horne & Reed (52) found that the high-affinity methotrexate system in rat hepatocytes was inhibited by the stilbene compound DIDS and a variety of unrelated organic and inorganic anions and was electrogenic, as indicated by stimulation following valinomycin-imposed K^+ potential across the membrane. Using the same vesicle system from rat and human hepatocytes, Horne et al (53, 54) showed that the high-affinity 5-methyltetrahydrofolic acid system was also located in the basolateral membrane. This system proved not to be sodium dependent, was not inhibited by anions, and was electroneutral and stimulated by an outwardly directed proton gradient. These authors concluded that the high-affinity methotrexate transport system appears to be a multispecific anion system in contrast to the more folate compound–specific, high-affinity 5-methyltetrahydrofolate system (48, 51–53).

CHOROID PLEXUS In view of the concentration gradient (105) of 5-methyltetrahydrofolate in cerebrospinal fluid versus plasma, it was not surprising to find that choroid plexus in vitro from hogs (107) and rabbits (105, 106) exhibited a system for transporting this folate into cerebrospinal fluid. The system in each case exhibited very high affinity for this folate (105, 107) and was also capable of transporting 5-formyltetrahydrofolate, folic acid, and the folate analogue methotrexate. The system in hog choroid plexus exhibited a similar affinity for 5-formyltetrahydrofolate but somewhat lower affinity for folic acid and methotrexate (107). In contrast, the system in rabbit choroid plexus appeared to have greatest affinity for folic acid compared with 5-methyltetrahydrofolate and least affinity for methotrexate (105, 106). It is unclear as to whether these differences are methodological or species dependent.

Membrane Systems Identified in Absorptive Epithelial Cells

LUMINAL EPITHELIUM OF SMALL INTESTINE From the very early work on folate absorption, reviewed by Selhub et al (91), it was concluded that a mediated system was involved that was saturable and that exhibited structural and stereochemical specificity and acid pH dependence. The first detailed study of this absorptive process documenting carrier mediation was done by Selhub & Rosenberg (93) using rat intestinal brush-border membrane vesicles isolated from mature absorptive cells (enterocytes). Influx occurred within a broad pH range (pH 4–7) with an optimum of pH 5. In addition, the affinity of the system for folic acid, 5-methyltetrahydrofolate, and the folate analogue methotrexate was approximately the same, with values for influx K_m or K_i of 1–4 μM. These workers also demonstrated accelerative exchange diffusion (93) by this system.

Studies by Sirotnak et al (15, 103) documented the existence of a different system internalizing folate compounds in isolated luminal epithelial cells. These studies, initially using mixtures of immature and mature (absorptive) cells, documented influx of various folate analogues at pH 7.4 that was moderately saturable and variable in preferences among these compounds as permeants (15). Subsequent studies (103) extended these earlier studies (15) using methodology that allowed for the fractionation of the luminal epithelial cells into mature absorptive and immature proliferative cellular components. These studies showed that the same transport system operating under mild alkaline buffer conditions in the earlier study (15) operated at the same level in each cell type. When examined in the context of those of Selhub & Rosenberg (93), the results of these studies suggested that this low-affinity transport system operates not in the brush-border membrane but in the plasma membrane.

More recently, using the same fractionation procedure, Chiao et al (18) delineated mediated folate compound transport inward in these isolated luminal

epithelial cells as acid pH-dependent and non–pH-dependent components on the basis of their differential sensitivity to DIDS. pH dependence for the brush-border system was manifested as higher V_{max} for influx of folate compounds at acid pH. This pH-dependent brush-border influx component was relatively insensitive to inhibition by DIDS and highly saturable (influx K_m or K_i = 2–4 μM) in the case of folic acid, folate coenzymes, and 4-aminofolate analogues as permeants or competitive inhibitors. The non–pH-dependent component was highly sensitive to DIDS and poorly and variably saturable (influx K_m or K_i = 20 to >2000 μM) with respect to these same compounds. Only the pH-dependent component was developmentally regulated (18), showing much higher influx V_{max} in mature absorptive rather than immature proliferative crypt cells. Treatment of mature absorptive cells with either a folate-based affinity label or antibodies against transporter-related peptides deduced from the RFC-1 (reduced-folate carrier) gene coding sequence (see below) inhibited influx of folate compounds (18). These results strongly suggested that the pH-dependent system associated with folate absorption in this tissue, originally described by Selhub & Rosenberg (93), is regulated by RFC-1 gene expression.

COLONIC EPITHELIAL CELLS IN CULTURE A system in the outer membrane of human NCM 460 cells, a normal colonic epithelial cell line recently established in culture, exhibited an acid pH optimum (pH 5) and was also highly saturable (influx K_m = 2–5 μM) for folic acid, 5-substituted reduced folates, and methotrexate (62). Indirect evidence was also presented in this report (62) that suggested that this transport system, which was similar to that found in small intestine, was regulated at least partially through the action of protein tyrosine kinase.

Membrane Systems Identified in Subcellular Organelles

LYSOSOMES Studies by Barrueco et al (4–6) identified a carrier-mediated transport system internalizing 4-aminofolyl polyglutamates and, ostensibly, folyl polyglutamates into lysosomes derived from murine tumor cells. These γ-glutamyl peptides of these folate compounds result from their rapid metabolism by folyl polyglutamate synthetase in the cytosol (reviewed in 4–6). This system exhibited greater affinity for the longer chain (three to four additional glutamates, influx K_m = 42 ± 8 μM) than for the shorter chain (one to two additional glutamates, K_m = 334 ± 19 μM) polyglutamates and did not internalize unpolyglutamylated folate compounds (4). Further studies showed that influx of these 4-aminofolyl polyglutamates exhibited a mildly alkaline pH optimum and a Q_{10} (27°–37°C) of 3.1 ± 0.1 (5). Moreover, exchange diffusion could be demonstrated that was accelerative for influx but decelerative for efflux with 4-aminofolate polyglutamates as permeants. This carrier-mediated

mechanism exhibited broad specificity for a large variety of folyl and 4-aminofolyl polyglutamates as well as α- or γ-glutamyl peptides and heteropeptides that have a C-terminal glutamate. This transport system limits, along with the internalization of cysteine, hydrolysis of these polyglutamates by a sulfhydryl activated enzyme, folyl polyglutamate hydrolase (γ-glutamyl hydrolase) (6).

MITOCHONDRIA Early studies provided evidence for the internalization of oxidized folates and the folate analogue methotrexate, but not reduced folates, into mitochondria (21). More recent studies (51), however, showed that a carrier-mediated system did operate within the mitochondrial membrane for internalizing 5-formyltetrahydrofolate. After internalization this folate is required for the metabolism of glycine in this organelle. This system was acid pH dependent and exhibited relatively high saturability (influx $K_m = 2$–8 μM) for 5-substituted tetrahydrofolates but less so for folic acid and methotrexate (51).

PROPERTIES OF THE ONE-CARBON, REDUCED-FOLATE TRANSPORTER

Molecular Mass Determinations

Studies from several laboratories (46, 79, 90, 124) have focused on the molecular mass of the one-carbon, reduced-folate transporter in the plasma membrane of murine tumor cells. The initial study (46), which utilized ligand affinity labeling of the transporter with the N-hydroxy-succinimide (NHS)-ester of [^{3}H]methotrexate and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), estimated it at 36 kDa in L1210 cells. However, three subsequent studies utilizing photoaffinity labeling (79) or affinity labeling (90, 124) with folate analogues and SDS-PAGE or molecular sieve chromatography derived a value of 46 kDa in the same cell type. This discrepancy in molecular mass involving one of the four studies was explained by showing that the conditions utilized in the initial study (46) were inadequate to suppress proteolysis of the transporter during its isolation prior to SDS-PAGE (124). This latter study also provided evidence that the transporter was a true integral membrane protein (124). Proof that the 46-kDa protein was the mediator of one-carbon, reduced-folate transport was obtained by showing that the NHS ester of 5-substituted reduced folates, but not of folic acid, effectively affinity labeled this protein and inhibited influx of folate compounds (79, 90, 124).

Subsequent studies showed by immunoblotting with polyclonal antibodies against the purified transporter that the same 46-kDa protein was found in the plasma membrane of other murine tumor cells (19, 83). The cloning of the gene (see below) encoding the one-carbon, reduced-folate transporter has provided information on the deduced amino acid sequence of this transporter.

Using antibodies raised against synthetic peptides encoded by this gene, Chiao et al (18, 83) were also able to identify by immunoblotting a 46-kDa protein in the plasma membrane of various murine tumor cells. NHS-[^{3}H]methotrexate affinity or photoaffinity labeling and SDS-PAGE were also used to estimate the molecular mass of this transporter in human tumor cells. In the plasma membrane of HL-60 (121) and K562 (70) leukemia cells, it was estimated to be 80–85 kDa. The same criteria used to identify the plasma membrane protein in murine tumor cells as the one-carbon, reduced-folate transporter (79, 90, 124) were used to identify the 80- to 85-kDa protein as the corresponding human transporter in these studies (70, 121). More recently, a plasma membrane protein of similar mass was found in CCRF-CEM (derived from lymphoid leukemia) cells with antibodies prepared against the purified murine (19, 69) or human transporter (69). Additional confirmation as to the identity of this 80- to 85-kDa protein was obtained (19, 69) by showing that the amount of this protein detected was commensurately higher or lower in CCRF-CEM cell variants with either high or low one-carbon, reduced-folate transport compared with parental cells. Also, coordinate down-regulation of this protein and one-carbon, reduced-folate transport occurred (121) in HL-60 cells induced to differentiate.

Biochemical Aspects

Two separate reports showed that the one-carbon, reduced-folate transporter in L1210 cells, unlike most integral membrane proteins, was unglycosylated (19, 28). In contrast, the corresponding transporter in human K562 and CCRF-CEM tumor cells was found to be heavily glycosylated (68, 69). Approximately 30–35% of the total mass of this protein was shown to be carbohydrate, with the majority representing N-linked glycosides. It is interesting that these carbohydrates appeared to play no role in regard to the function of this transporter (68). This was shown by inhibiting glycosylation following growth of CCRF-CEM cells in the presence of tunicamycin and examining folate influx (68). Evidence was obtained in other studies that suggested that the rate of one-carbon, reduced-folate transport (influx velocity) responded to alteration in intracellular folate homeostasis (57). In these studies, it was shown that influx of [^{3}H]methotrexate was markedly reduced when CCRF-CEM cells were grown in culture in hyperphysiological 5-formyltetrahydrofolate concentrations (57). By ligand affinity labeling, it was found that the level of the transporter was unchanged in cells grown at different medium concentrations of folate (57). Thus, it was concluded that the rate of translocation of the carrier (transporter) must vary in a manner that was dependent on the concentration of folate within the cell (57). The biochemical/molecular basis for this form of regulation of one-carbon, reduced-folate transport has not yet been elucidated.

MOLECULAR BIOLOGY OF ONE-CARBON, REDUCED-FOLATE TRANSPORT

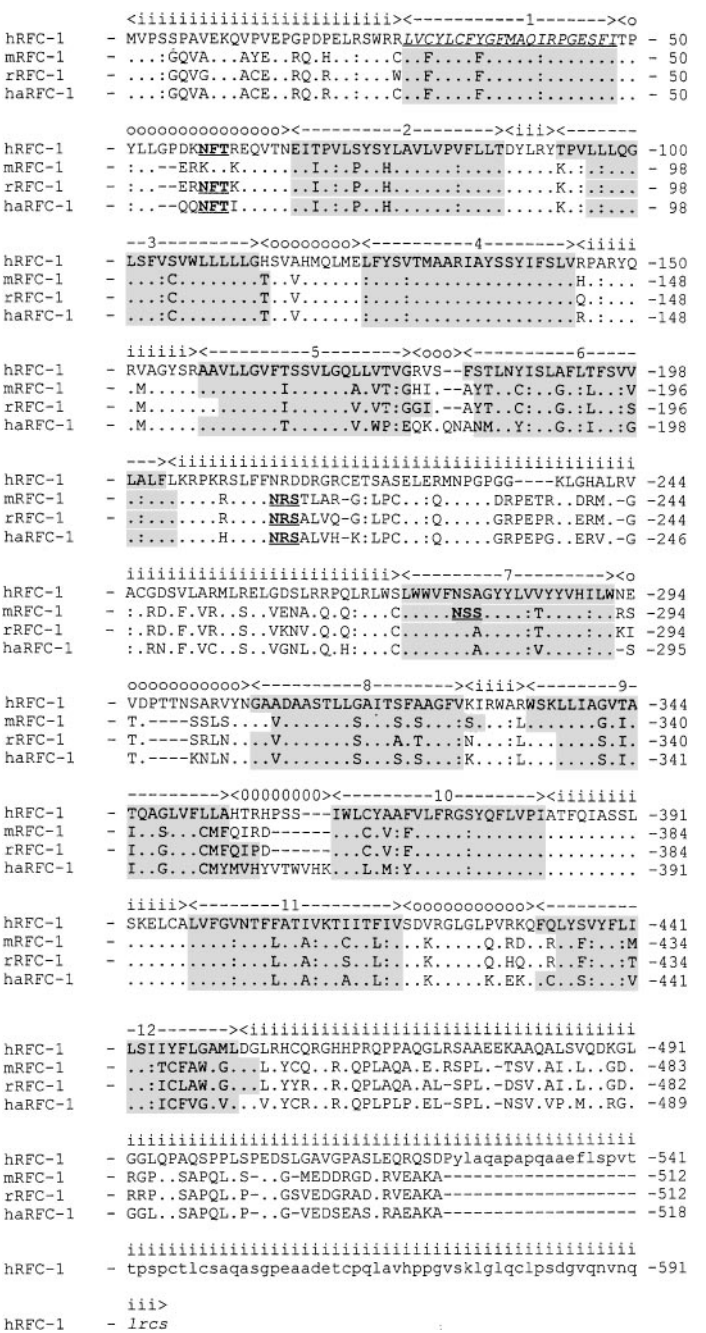
cDNA Cloning of the RFC-1 Gene

Molecular cloning based on complementation by DNA-mediated gene transfer of a methotrexate resistant Chinese hamster ovary cell line with a major deficit in folate transport yielded human as well as hamster DNA fragments that could restore MTX transport (113, 114). Subsequently, murine (24), hamster (118), and eventually rat (GenBank/EMBL Accession #U38180) and human (72, 74, 78, 117, 119) cDNA clones were isolated that conferred on transfection to methotrexate-resistant, transport-deficient cell lines many of the properties of the one-carbon, reduced-folate transport. More definitive proof that the RFC-1 (reduced-folate carrier) gene encodes the one-carbon, reduced-folate transporter relies on findings discussed below. In these studies, it was shown that nucleotide alterations within the open reading frame (ORF) of the murine (m) RFC-1 cDNA affected the properties of one-carbon, reduced-folate transport in murine tumor cells or their RFC-1 cDNA transfectants (11, 39, 83, 127, 128). Screening for coding sequence of the various cDNA clones revealed that the ORF encoded 58-, 58-, 59-, and 65-kDa proteins in murine, rat, hamster, and human cDNA, respectively (24, 72, 74, 78, 117–119; GenBank Accession #U38180). The estimated pI of these proteins was approximately 9. The individual sequences reveal a number of potential N-glycosylation sites. However, only N^{58}, N^{56}, and N^{56} in human (h), hamster (ha), and rat (r) RFC-1, respectively, are located in an extramembrane loop (see below) and could contribute to N-glycosylation (Figure 1). This is consistent with the lack of N-glycosylation found in the case of the murine transporter (see above). Alignment of the published amino acid sequences (24, 72, 74, 78, 117–119; GenBank Accession #U38180) indicates that the extent of amino acid homology varies between 65–90% (Table 2) and that identical or similar residues are distributed along

Table 2 Amino acid identity (similarity) between pairs of transporters[a]

	Amino acid identity (similarity) with (%)			
Transporter	hRFC-1	mRFC-1	haRFC-1	rRFC-1
hRFC-1	100			
mRFC-1	66.0 (9.4)	100		
haRFC-1	65.3 (8.7)	80.4 (5.7)	100	
rRFC-1	65.2 (8.4)	89.7 (2.7)	83.4 (4.5)	100

[a]Alignment was performed by computer-assisted analysis with PCGene software (release 6.26, Genofit, Geneva, Switzerland). The best fit was achieved by introducing gaps in order to maximize the identity score. The aligned sequences are human (h) (72, 74, 78, 117, 119), mouse (m) (24, 86, 110), hamster (ha) (18), and rat (r) (GenBank/EMBL Accession #U38180) RFC-1 (reduced-folate carrier).



the entire amino acid sequence (Figure 1). The number of membrane-spanning helices of the proteins indicated in Figures 1 and 2, as predicted according to Eisenberg et al (27), is 12 for the rodent and 11 for the hRFC-1. The hydropathy profiles and the location of the predicted membrane-spanning helices are also similar (63) when comparing the RFC-1 proteins to each other. This observation makes it plausible that hRFC-1 also has 12 membrane-spanning helices (Figures 1 and 2). The proposed transmembrane segment 1 appears to be amphipathic in all RFC-1 proteins. According to general topology rules for eukaryotes, viz the positive inside rule (47, 77, 95), the N and C termini of the RFC-1 transport proteins are intracellular (Figures 1 and 2). The location of extra- and intracellular loops is also indicated (Figures 1 and 2). An extension of the C terminus reflects the larger size of the human protein (Figure 1). A major discrepancy emerging from some of these molecular studies (12, 24, 109) and from earlier biochemical studies (75, 90, 124) that remains unexplained pertains to the molecular mass of the murine transporter encoded by RFC-1. The ORF predicts 58 kDa, whereas that estimated by SDS-PAGE in murine tumor cells is 46 kDa. This discrepancy may not be explained simply by aberrant electrophoretic migration because the same mass was estimated during molecular sieve chromatography and RFC-1 encodes the same transporter (18) in luminal epithelium of mouse small intestine that migrates as 58 kDa during the same SDS-PAGE.

Genomic Organization and Chromosomal Localization of the RFC-1 Gene

Data on the complete genomic organization and structure have now been reported for ha- (73), m- (12, 109), and hRFC-1 genes (110). They are summarized in Figure 3. The hamster gene is composed of seven primary exons, whereas the murine and human genes are both composed of six primary exons with one and four alternates of exon 1 in the murine and human gene,

←

Figure 1 Alignment of human (h), murine (m), rat (r), and hamster (ha) RFC-1 (reduced-folate carrier). Gaps in the sequences were introduced to maximize the identity score. (*Shading*) Membrane-spanning fragments, as predicted according to Eisenberg et al (27); (*lowercase, lowercase/italics*) amino acid residues not encoded or additionally encoded, respectively, by hRFC-1 cDNA KS32 (119) relative to other reported hRFC cDNAs; (*italics/underlined*) putative first helix of hRFC-1; (*bold/underlined*) tripeptide encoding putative N-glycosylation site (see text); (*dots* and *colons*) identical and similar residues, respectively (similar groups: A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y; W); <iii>, internal loops; <ooo>, external loops; >, proposed membrane-spanning segment. Because all cDNAs reported show minor variations in polypeptide sequence, sequences aligned for h-, m-, r-, and haRFC-1 are taken from Wong et al (119), Dixon et al (24), database entry U38180, and Williams et al (118), respectively.

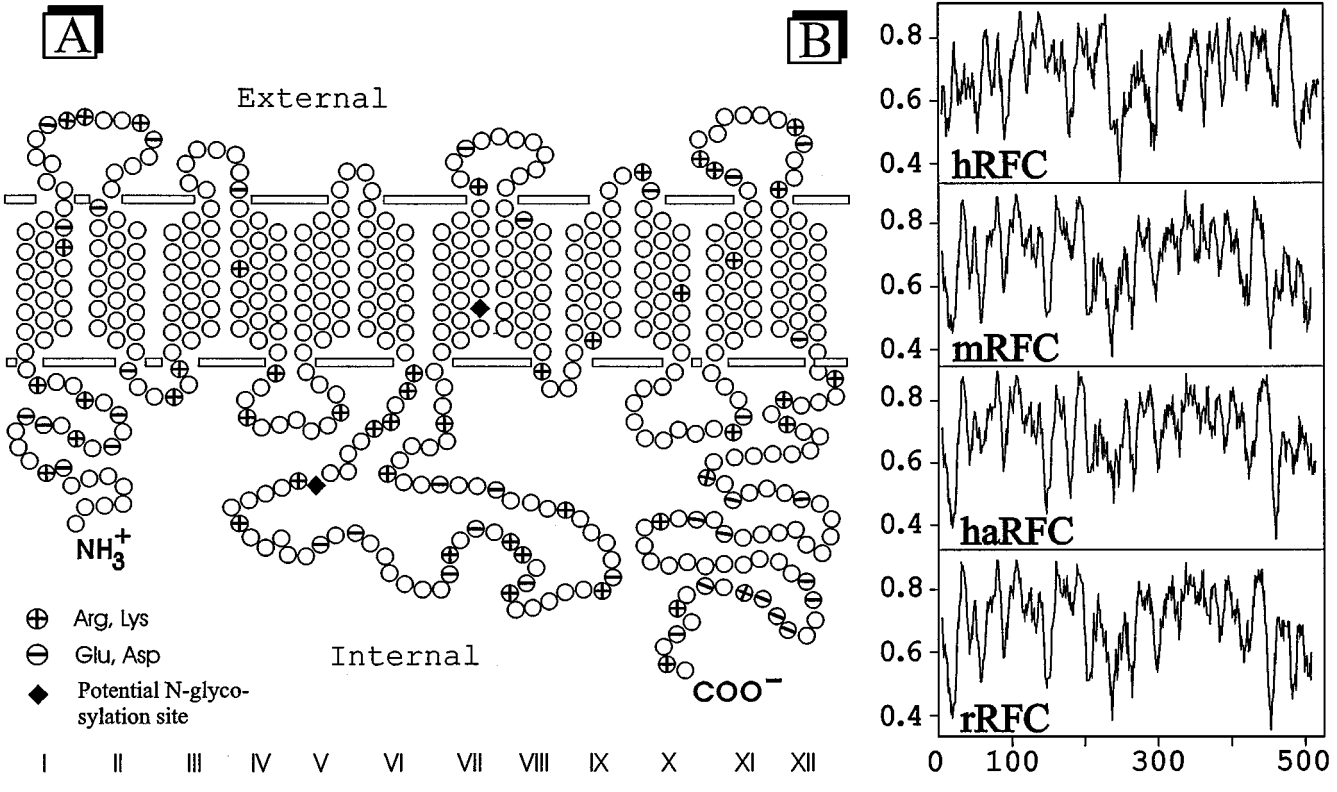


Figure 2 (*A*) Secondary structure model of murine (m) RFC-1 (reduced-folate carrier) and (*B*) (normalized) hydropathy profiles of the human (h), rat (r), hamster (ha), and mRFC-1 transport proteins. The hydropathy was calculated according to the method of Eisenberg et al (27), with a window of 21 amino acids. The positions of the 12 membrane-spanning segments are indicated by Roman numbering. Charged residues and potential glycosylation sites are indicated.

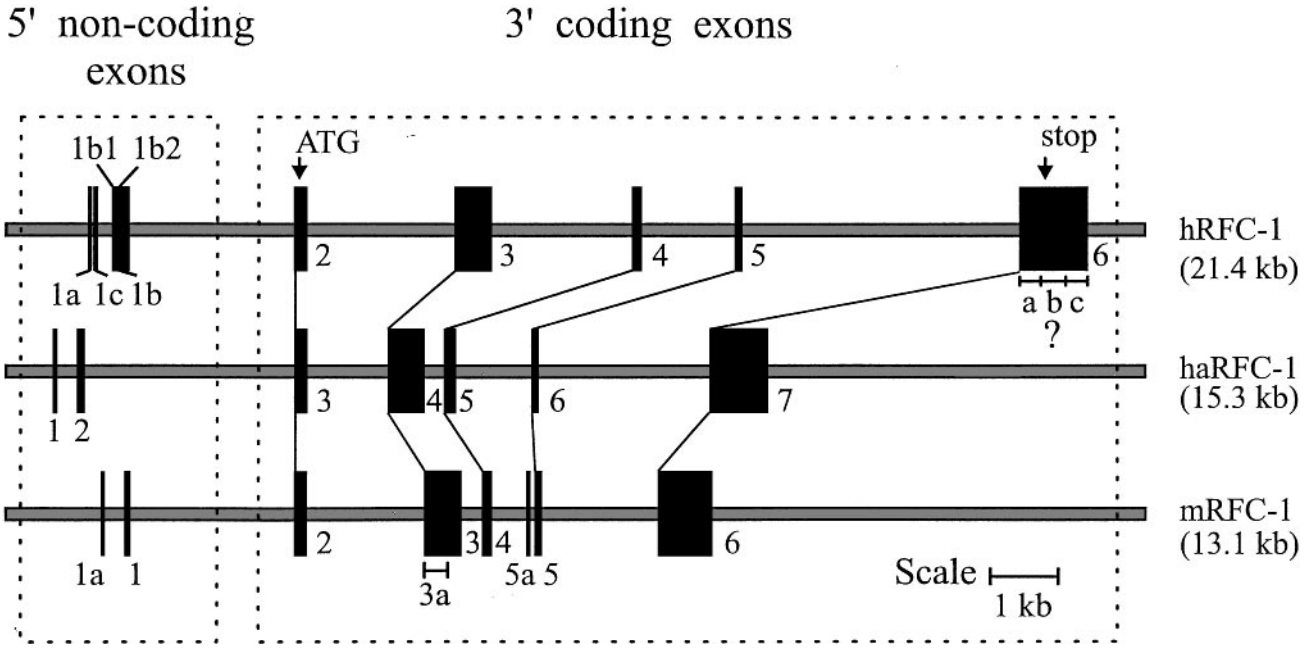


Figure 3 Intron and exon composition and length of human (h), murine (m), and hamster (ha) RFC-1 (reduced-folate carrier) gene. The genomic sequences are drawn to scale and ha- and mRFC-1 are aligned relative to exon 2 of hRFC-1. (*Solid lines*) Homologous exons. The translational start and stop codons are indicated.

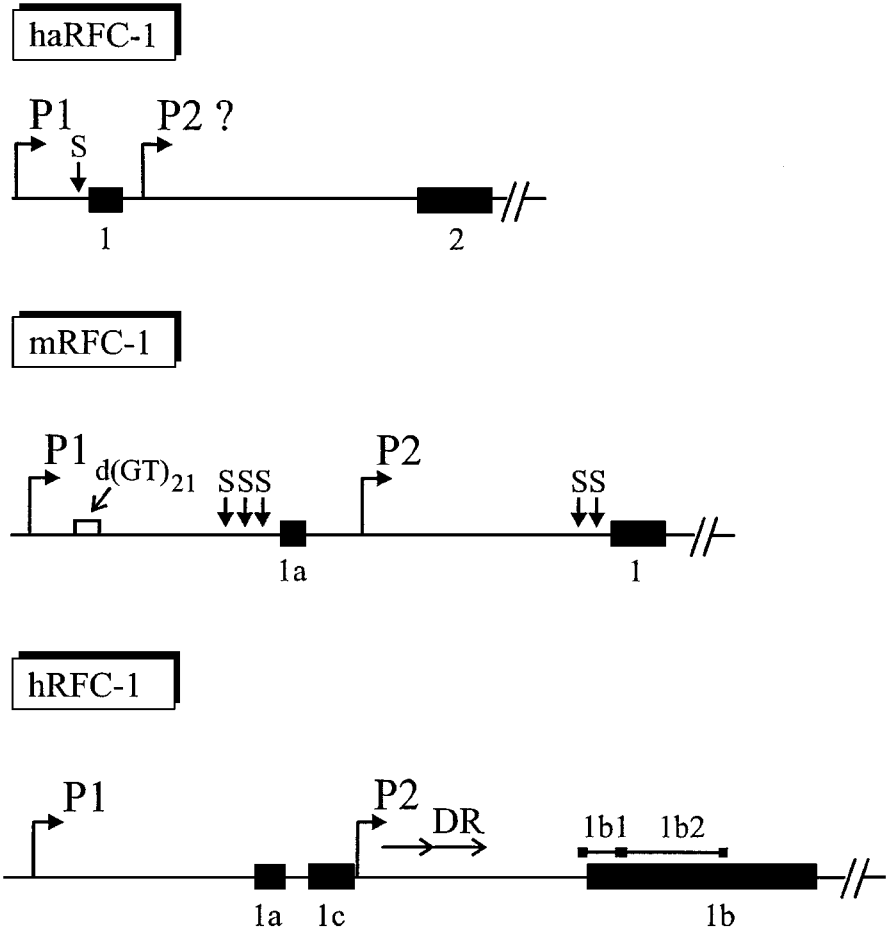
respectively. Comparison of the primary coding exons in hRFC-1 (110), mRFC-1 (12, 109), and haRFC-1 (73)—exons 2–6, 2–6, and 3–7, respectively—reveals that the positions of the splice junctions yielding these primary exons are almost perfectly conserved, resulting in exons of similar length (Figure 3). In addition, most of the intron/exon splice junctions conform to the GT-AG rule (76). Furthermore, the position of start and stop codons can be found in homologous exons, i.e. exon 2 (or 3 in case of haRFC-1) and exon 6 (or 7 in case of haRFC-1), respectively (Figure 3). In contrast to exon length, considerable differences are found among these genes in terms of intron length, resulting in overall gene lengths of 21.4, 15.3, and 13.1 kb for h-, ha-, and mRFC-1, respectively. Various techniques, including human-rodent somatic cell hybrid analysis, in situ hybridization using metaphase chromosomes, and fluorescence in situ hybridization (FISH), assigned hRFC-1 to chromosome 21 at region 21q22.2-3 (72, 120, 125). In addition, hRFC-1 is precisely localized (64) on a high-resolution map spanning 2.5 Mb of region 21q22.3 and appears to be in close association with the α1 collagen gene (COL18A1). Based on genomic nucleotide sequencing, the mRFC-1 gene was assigned to chromosome 10 (82), 2 kb downstream of the same collagen gene (COL18A1) at locus 38.3 g (81). haRFC-1 was assigned to chromosome 1 at position q2 $\rightarrow$ q3 (13).

RFC-1 mRNA Splice Variants

The most important difference between the m-, ha-, and hRFC-1 transcripts is their degree of 5′ end heterogeneity (Figure 4). The haRFC-1 transcripts display no 5′ end heterogeneity (73), and the corresponding gene consists of 7 exons with the additional exon (exon 2) encoding 5′ untranslated region (UTR) sequences along with exon 1 in a contiguous manner. In contrast, the mRFC-1 transcript heterogeneity is related to the existence in the gene of a true alternate to exon 1 (12, 109), whereas the hRFC-1 transcript heterogeneity appears to be related to far more complexity in this gene, which has four alternates to exon 1 (110, 126).

HRFC-1 SPLICE VARIANTS So far, five RFC-1 transcripts have been identified in various human cDNA libraries with different 5′ UTRs but encoding the same transport protein (72, 74, 78, 117, 119). Genomic sequencing (110, 126)

Figure 4 The 5′ organization and exon composition of splice variants of hamster (ha), murine (m), and human (h) RFC-1 (reduced-folate carrier) genes. P1 and P2, promoter 1 and 2 respectively; S, SP1 site; DR, direct repeat; d(GT)21, dinucleotide repetitive element. (From 12, 24, 72–74, 78, 86, 109–111, 117–119, 126.)



exon composition	var. #
1 2 3 4 5 6 7	I
2 3 4 5 6 7	II

1 2 3 4 5 6	I
1 2 3a 4 5 6	II
1 2 3 4 5a 6	III
1a 2 3 4 5 6	IV

1a 2 3 4 5 6	I
1b 2 3 4 5 6a6b	II
1b1 2 3 4 5 6	II.1
1b2 2 3 4 5 6	II.2
1c 2 3 4 5 6	III

showed that these transcripts are a result of alternate splicing of RNA transcripts from the same gene (Figure 4). Variants I and II incorporate exon 1a or 1b, respectively, and exons 2 through 6 (110, 126). The identification of variants I and II as splice variants is consistent with the ability of their cDNA to restore folate transport (119) following transfection of a transport-defective cell line. Variant II incorporates sequence homologous to exon 6 but with an internal segment of 626 nucleotides (119) in length deleted, which suggests that exon 6 actually consists of three exons (exons 6, 7, and 8) (Figure 3). On the protein level, this means a deletion of 67 intracellularly located (Figure 2) C-terminal amino acid residues relative to variant I, resulting in a somewhat smaller mass (58 kDa versus 65 kDa for variant I). According to 5′ RACE (rapid amplification of cDNA ends), two other variants, variant II.1 and II.2, appear to consist of a different 5′ region of exon 1b (126). Variant III (119) appears not to be full length and incorporates (*a*) another alternate to exon 1 (exon 1c), (*b*) exon 2, and (*c*) a truncated exon 3. According to the genomic (and 5′ end) organization of hRFC-1 (110, 126), the previously cloned human placental folate transporter, hFOLT (78), and the intestinal folate transporter, hIFC-1 (74), appear to be RFC-1 splice variants (variant I). However, these cDNAs encode proteins with a conserved N-terminal region, but highly diverse C-terminal region, relative to each other and to those cloned in other laboratories (72, 74, 78, 117, 119). This may be the result of discrepancies in the sequencing reported.

MRFC-1 SPLICE VARIANTS As is the case with hRFC-1, all mRFC-1 transcripts reported so far (12, 86, 109) are, according to genomic sequence analysis (12, 109), a result of splicing of RNA transcripts from the same gene (Figures 3 and 4). Variant I consists of exon 1 plus exons 2 through 6 encoding a polypeptide of 58 kDa (12, 109). Variant II also consists of exon 1 plus exons 2 through 6. However, in this variant, 120 bp of cDNA was deleted at the most 3′ end of exon 3 (109). This "in frame" deletion reduces the encoded polypeptide to 53.6 kDa. A similar variant (variant II.1) is described (11) but carries a 119-bp instead of a 120-bp deletion, making the encoded polypeptide 40 kDa. A third variant (variant III) comprises exon 1 plus exons 2–4, and an apparent alternative to exon 5 (exon 5a) in addition to exon 6, encoding a polypeptide of 43.4 kDa (12). Variant IV (86) incorporates an alternate to exon 1 (exon 1a) but is otherwise similar in composition to variant I. Variants II and III encode only 11 and 10 membrane-spanning regions, respectively, compared with 12 encoded by variants I and IV. In addition, variant II and III (109) and other variants with various other internal deletions (12) appear to be rare, and their significance is unknown. Screening of an L1210/R83 cDNA library (109) and quantitative reverse transcriptase–polymerase chain reaction on an L1210 mRNA isolate (111) revealed that variant I is the most abundant splice form in this tumor,

whereas variant IV appears to be the most prominent species in mouse small intestine (111). The latter suggests a difference in transcriptional regulation with respect to the formation of the RFC-1 transcripts in human and murine intestine, because in mice, a unique variant (variant IV) is produced in the intestine (86, 111). This is in contrast to the situation in humans, where variant I is the only major transcript reported so far.

HARFC-1 SPLICE VARIANTS The hamster gene, in comparison to mRFC-1 and hRFC-1, has only 2 splice variants (73) (Figure 4). Variant I is composed of exons 1 through 7, whereas variant II consists of exons 1 and 3 through 7. Exons 1 and 2 contain the 5′ UTR, whereas the ATG start codon can be found in exon 3. Thus, the encoded proteins are identical. The frequency of each variant at least appears to be the same in Pro^{-3} Chinese hamster ovary cells (73).

Regulation of Transcription and Promoter Multiplicity

The 5′ organization of m (109), ha- (73), and hRFC-1 (110, 126) genes is similar in that in each there is a large (3–3.5 kb) intron upstream of the exon carrying the ATG start codon (Figure 4) that includes alternates to exon 1 (Figures 3 and 4). Furthermore, these alternative exons have 5′ flanking regions that are TATA-less but that in the case of mRFC-1 (109) and hRFC-1 (110, 126) carry numerous SP1 sites, whereas the information about haRFC is limited but the data reveals at least one consensus SP1 site (73). The 5′ heterogeneity of the RFC-1 transcripts suggests the involvement of multiple promoters.

HRFC-1 PROMOTERS The 5′ flanking regions of the genomic DNA upstream of the alternative exon 1 is highly GC rich and contains many putative transcription factor binding sites for such transcriptionally involved proteins as MZF1, AP1, AP2, and SP1 (110, 126). In addition, the 5′ flanking region of exon 1b contains a direct repeat sequence (110, 126). Transfection studies of Chang's Liver (CL) and SK-Hep-1 cells (110) with constructs of putative promoter regions fused to a luciferase reporter gene reveal the involvement (Figure 4) of at least two separate promoters in transcription of the hRFC-1 splice variants, i.e. the 5′ flanking region of exon 1a (P1), as well as that of exon 1b (P2). Basal P1 activity in CL and SK-Hep-1 cells (110) and HT1080 cells (126) is confined to a 5′ flanking region of exon 1a of 594 and 360 bp, respectively, whereas in the same cells P2 activity is sustained within a sequence of about 240 nucleotides directly flanking exon 1b (110, 126). In Cl and SK-Hep-1 cells (110) and in HT1080 cells (126), P1 appears to be less active compared with P2. The activity of P1 was increased severalfold in promoter/reporter constructs containing an SV40 enhancer element (110), which suggests involvement of additional features that cause these promoters to differentially express hRFC-1 splice variants in different tissues and/or cell lines. In Cl and SK-Hep-1

cells (110), the differences in promoter efficiency are consistent with splice variant–specific primer extension. In addition, primer extension analysis of the region upstream of exon 1a and 1b in SK-Hep-1 and CL (110) as well as in CCRF-CEM and K562.4CF cells (126) reveals numerous transcriptional start sites, which is consistent with the multiplicity of SP1 sites and with the absence of TATA-like consensus sequences.

MRFC-1 PROMOTERS The 5′ flanking region of mRFC-1 (109) exons 1a and 1 are relatively GC rich and contain many putative Ets-1 and SP1 binding sites. In addition, two inverse CCAAT boxes and a dinucleotide repetitive element are located (Figure 4) upstream of exon 1a. Promotor multiplicity (Figure 4) of mRFC-1 was confirmed by functional deletion analysis and site-directed mutagenesis of 5′ flanking regions of exon 1a (promoter 1, P1) and 1 (promoter 2, P2), fused to a reporter gene in constructs transfected into NIH3T3 cells (111). The promoter activity of P2 appears to be approximately fivefold higher than P1 in these constructs. However, the activity of P1 was severalfold increased on cloning in constructs containing an SV40 enhancer element. Basal promoter activity of P1 and P2 is confined to a region of 123 and 60 bp, respectively, directly 5′ of the respective exons. In P1, this region contains three SP1 sites, of which the middle site is the most important, as was shown by site-directed mutagenesis studies. A poly$(GT)_{21}$ dinucleotide repetitive element was found just upstream of these three SP1 (109) sites and has suppressor activity (111). The smaller P2 contains two SP1 sites that are equally important. The role of each SP1 site in maintaining basal activity of each promoter was confirmed by DNase I footprinting analysis. In addition, tissue-specific expression in the form of RFC-1 mRNA splice variants reflects the separate action of each promoter (see prior section).

Mutational Alterations

Alterations in the functional properties of the murine one-carbon, reduced-folate transporter as a result of nucleotide differences in the Open Reading Frame (ORF) have recently been reported from three laboratories (11, 83, 112, 127, 128). These nucleotide alterations affect the rate of translocation of this membrane protein (11, 128), its interaction with folate compounds (83, 112) as permeants, or both (127). Some of these alterations were identified in L1210 cell variants made resistant to methotrexate or other folate analogues. An Ala to Pro substitution as a result of a heterozygous mutation (G → C at nucleotide +429) was found (11) within the fourth predicted transmembrane domain, which reduced influx V_{max} for folate compounds in a resistant variant 100-fold. Roy et al (83) reported on a Ser to Asn difference at position 297 between the seventh and eighth membrane-spanning domains, which occurred in sarcoma

180 cells as compared with L1210 cells. This difference, which was due to a heterozygous nucleotide alteration (G → A at +890), accounted for a highly selective effect on the interaction of the transporter with folate analogues bearing only an alkyl substitution at NIO. A homozygous mutation (G → A at +137) was identified in an L1210 cell variant (128) that evoked a Ser to Asn change occurring within the first predicted membrane-spanning domain. This amino acid substitution resulted in a marked decrease in influx V_{max} for methotrexate and to a lesser extent for 5-formyl and 5-methyltetrahydrofolate. A second nucleotide alteration (G → A at +133) was also identified in another variant within the first transmembrane domain that evoked a Glu to Lys change at position 45 (127). Influx V_{max} (12-fold) and K_m (fourfold) were both decreased for 5-formyltetrahydrofolate. However, influx K_m was increased for methotrexate (sevenfold) but decreased (sevenfold) for folic acid. In addition, influx V_{max} for methotrexate and 5-formyltetrahydrofolate, but not folic acid, was increased in the presence of inorganic anions. The results of these two studies appeared to suggest that mutational alterations of carrier mobility might be permeant specific and that this specificity could be modified by other anionic interactions with the carrier. Most recently, Tse et al (112) documented two heterozygous point mutations in the RFC-1 gene of an L1210 cell variant resistant to 5,10-dideazatetrahydrofolate. These mutations were identified in codons 48 (Ile48Phe) and 105 (Trp105Gly) and were the result of A to T (+183) and T to G (+354) transversions, respectively. Each of these mutations contributed to a large increase in the affinity of the transporter for folic acid, but not 5-formyltetrahydrofolate, and to a small decrease in affinity for the analogue. It is surprising that the amino acid substitutions that bring about these selective effects are within the first (Ile48Phe) and third (Trp105Gly) membrane-spanning helices rather than at an externally located position. Mutations affecting functional expression of RFC-1 were also recently identified (39) in codons 25 and 40 of the ORF of this gene in MTX-resistant MOLT-3 cells, which resulted in premature peptide chain termination. This occurred following introduction of premature stop codons by G to A and C to T transitions of nucleotides +73 and +117, respectively.

RFC-1 Expression and Intestinal Folate Absorption

Recent studies (18, 86) have shown that acid pH-dependent folate transport (see above) in the luminal epithelium of mouse small intestine is due to the expression of the RFC-1 gene. Moreover, expression of this gene was developmentally regulated, with expression occurring (18, 86) predominantly in the mature absorptive cells. The most convincing evidence provided (18) showed that a specific affinity label and anti–RFC-1 peptide antibodies inhibited acid pH-dependant folate transport. These were surprising findings, because properties

of RFC-1–mediated folate transport in these cells are markedly different (see above) from tumor cells. The biochemical basis for this difference in functional expression of this gene in each cell type remains unknown. It should also be pointed out that RFC-1 was similarly expressed in luminal epithelium of human small intestine (74).

CARRIER-MEDIATED FOLATE TRANSPORT AND FOLATE HOMEOSTASIS

Developmental Regulation of Folate Transport

It has been proposed in the context of earlier reports (reviewed in 7, 59) that one way in which proliferative cells may regulate their macromolecular biosynthesis is through the regulation of folate homeostasis. In addition to the metabolic interconversion of folate compounds, this could occur at the level of mediated entry of exogenous folates. As one approach to understanding the manner by which the expression of this property is regulated in proliferative cells, the expression of one-carbon, reduced-folate transport in the plasma membrane during induced maturation of murine erythroleukemia and HL-60 promyelocytic leukemia cells has been examined (20, 96, 101, 121). These studies showed that the level of inward transport of folates and the synthesis of the transporter encoded by the RFC-1 gene rapidly declined during induced maturation of these cells. That this apparent down-regulation of folate transport was a result of cellular maturation per se was indicated by the fact that it proceeded growth arrest and was irreversible once cells were committed to a program of terminal maturation (101, 121). More recent studies (25) provided evidence for the notion that decreased folate homeostasis resulting from down-regulation of folate transport as well as dihydrofolate reductase (101) and folylpolyglutamate synthetase (25) actually contributed to the onset of terminal maturation of HL-60 cells. This evidence was obtained by showing that the onset of maturation following exposure to inducer was dependent on folate status and was delayed in high folate concentrations and markedly delayed under conditions that obviated the requirement of folate for growth (25). These results suggested that early down-regulation of these and, possibly, other folate anabolic properties is programmed to limit macromolecular biosynthesis and, thus, facilitate the switch from a program of proliferation to one of terminal maturation (25).

Requirement for Tumor Cell Growth and Leukemogenesis

Although it has been generally assumed that some capacity for intracellular accumulation of exogenous folate cofactors from biological fluids is normally required for growth and survival of mammalian cells with proliferative capacity, the extent of this requirement has only been addressed in a single study. This

study (100) showed that only a relatively finite deficit (5- to 10-fold) in influx V_{max} for 5-formyltetrahydrofolate was tolerated in the context of growth in vitro of genetic variants in a physiological concentration of this folate. Further deficit of folate transport in other variants required increasing concentrations of this folate or supplementation with folic acid, which can utilize alternative entry routes (see above). Similarly, this study (100) showed that tumor cell growth and leukemogenesis in mice correlated inversely with the degree of the deficit in folate transport, and in some variants with essentially no transport capacity, growth did not occur at all. However, growth and leukemogenesis of such a variant could be brought about if mice were concurrently administered folic acid, but not 5-formyl or 5-methyltetrahydrofolate. These results would clearly affirm the notion that tumor cells, if not all proliferative cells, are unable to sustain in vivo any measure of growth or macromolecular biosynthesis by nucleoside and nucleobase salvage alone and require a minimum ability to internalize exogenous folates as well.

CONCLUSIONS AND PERSPECTIVES

Maintaining folate homeostasis is essential for sustaining macromolecular biosynthesis and cellular proliferative potential. In this regard, processes involved in internalizing folates play a key regulatory role at the level of both the cell and the whole organism. Carrier-mediated processes represent a major route for cellular internalization, epithelial cell absorption, and subcellular trafficking of folates. Although many of the functional and physiological features of carrier-mediated one-carbon, reduced-folate transport have been elucidated, definitive information is lacking pertaining to energetic considerations, the nature of the translocation mechanism, and the tertiary structure and multimeric nature (if any) of the transporter within the membrane. Similarly, although a substantial amount of information has recently been amassed pertaining to the RFC-1 gene and the molecular biology of the one-carbon, reduced-folate transporter it encodes, notions as to the regulation of its expression at transcriptional, translational, and posttranslational levels need further experimental refinement. This is needed particularly in the context of mRNA splicing and tissue-specific gene expression, embryogenesis, cellular differentiation, and neoplastic transformation. In addition, discrepancies remain pertaining to the actual and predicted molecular masses of the transporter and the manner by which RFC-1 gene expression is manifested with markedly different biochemical and physical properties in absorptive and nonabsorptive tissues, including neoplastic cells. However, with the information already at hand on the RFC-1 gene and regulation of its expression, it should be possible to address issues at a molecular level pertaining to functional deficits in humans in folate absorption that may

underlie folate deficiency as well as other aberrant regulatory responses associated with dietary and folate homeostatic disorders.

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