RFC-1 Gene Expression Regulates Folate Absorption in Mouse Small Intestine*

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Mediated folate compound transport inward in isolated luminal epithelial cells from mouse small intestine was delineated as pH-dependent and non-pH-dependent components on the basis of their differential sensitivity to the stilbene inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid. pH dependence was manifested as higher maximum capacity (V$_{max}$) for influx of L-5-CH$_3$-H$_4$folate at acidic pH compared with neutral or alkaline pH with no effect on saturability (K$_m$). The pH-dependent component was relatively insensitive to inhibition by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid and highly saturable (K$_m$ or K$_i$ = 2 to 4 μM) in the case of folic acid, folate coenzymes, and 4-aminofolate analogues as permeants or inhibitors. The non-pH-dependent component was highly sensitive to 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid and poorly and variably saturable (K$_m$ or K$_i$ = 20 to >2000 μM) with respect to these folate compounds. Only the pH-dependent transport component was developmentally regulated, showing much higher maximum capacity for L-5-CH$_3$-H$_4$folate influx in mature absorptive rather than proliferative crypt cells. The increase in pH-dependent influx during maturation was associated with an increase in RFC-1 gene expression in the form of a 2.5-kilobase RNA transcript and 58-kDa brush-border membrane protein detected by folate-based affinity labeling and with antimouse RFC-1 peptide antibodies. The size of this protein was the same as that encoded by RFC-1 mRNA. The treatment of mature absorptive cells with either the affinity label or the anti-RFC-1 peptide antibodies inhibited influx of L-[H]5-CH$_3$-H$_4$folate in a concentration-dependent manner. These results strongly suggest that pH-dependent folate absorption in this tissue is regulated by RFC-1 gene expression.

Transport of folate compounds into mammalian cells can occur via carrier-mediated (1–5) as well as receptor-mediated (3–7) mechanisms depending on the relative level at which each is expressed in the plasma membrane of these cells. Carrier-mediated mechanisms are involved (1–4) in the internalization of both natural folates and their cytotoxic analogues in tumor and normal proliferative tissues. Thus, their level of expression in these tissues has pharmacological significance. Carrier-mediated transport of folate compounds among tumor cells from various tissues in different mammalian species appears to share (1–4) similar properties. They exhibit (1–4) similar diversity in saturability for various folate compounds and their analogues with extremely low saturability for folic acid compared with folate coenzymes. The properties of folate compound transport in normal tissues appear to be much more diverse (reviewed in Ref. 2) and often differ substantially from those characteristic of mechanisms found in tumor cells. Carrier-mediated transport of folates associated (8–11) with absorption in the small intestine of rodents appears to be uniquely different from that in tumor cells (1–4, 12), particularly in terms of its pH dependence and saturability. For instance, in absorptive luminal epithelium of the rat small intestine, a mechanism in the brush-border membrane has been described (11, 13) which exhibits an optimum at pH 5.5–6 and relatively high saturability (K$_m$ < 5 μM) for internalization of all folate compounds and their analogues examined, including folic acid. Another carrier-mediated mechanism transporting folate compounds has been described (14) in the luminal epithelium of mouse small intestine. This moderately saturable mechanism mediates high capacity influx and does not appear (14) to be associated with folate absorption. Since this mechanism is operable (14) at the same level in both absorptive and nonabsorptive proliferative tissue in the luminal crypt, it would appear to be localized in the plasma membrane at the basolateral surface.

Recently, a murine cDNA clone (RFC-1) was isolated (15) by expression cloning from an L1210 cell cDNA library. This cDNA appears to encode a protein with many of the properties of the tumor cell, 1-carbon reduced folate transporter. However, expression of the RFC-1 gene has also been shown (16) to occur in the small intestine of rodents and appears to be under developmental regulation at least in rat intestine. This was based on the finding in the rat of higher levels of RFC-1 mRNA in mature absorptive cells compared with that found in immature proliferative cells in the crypt. However, direct evidence showing that this gene does, in fact, regulate folate absorption in this tissue was not provided.

The present studies are an extension of these recently reported (16) and of our own earlier (14) studies of folate compound transport in luminal epithelial cells isolated from mouse small intestine. In these new studies, we were able to delineate by kinetic and other means acid pH-dependent influx of L-5-CH$_3$-H$_4$folate from non-pH-dependent influx mediated (14) by the mechanism that appears to operate in the plasma (basolateral) membrane of these cells. We also provide evidence showing that acid pH-dependent folate compound transport in brush-border membrane in mature absorptive cells is inhibited.

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The abbreviations used are: L-5-CH$_3$-H$_4$folate, the natural diastereomer of 5-methyltetrahydrofolate; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; NHS, N-hydroxysuccinimide.
by a folate-based affinity label and by antibodies against RFC-1-specific peptides. Folate transport in absorptive epithelial cells from the mouse also appears to be dependent on the expression of the RFC-1 gene (15) in the form of a 2.5-kilobase transcript and a 58-kDa transporter encoded by this transcript and detected by the same folate-based affinity label and by immunoblotting in the brush-border membrane of these cells.

EXPERIMENTAL PROCEDURES

Isolation of Luminal Epithelial Cell Fractions—The isolation of luminal epithelial cells from the small intestine of C57BL/6 x DBA/2 F$_1$ (BD2F$_1$) mice was carried out at 0–4 °C by a previously published (14) method. These cells were obtained in the form of four individual fractions varying in their stage of maturation using marker enzyme analysis (14) to monitor fractionation. The isolation of purified brush-border membrane from Fraction I luminal epithelial cells was the same as that described (13, 17) for rat luminal epithelial cells.

Transport Methodology and Affinity Labeling—Freshly isolated cells in Fractions I and IV were utilized in experiments measuring the mediated internalization at 37 °C of $L$-$[^{3}H]$-5-CH$_3$-H$_4$ folate by luminal epithelial cells from mouse small intestine. $A$, influx by Fraction I cells at pH 6.2 and pH 7.4. $B$, influx by Fraction I and Fraction IV cells at pH 6.2; $ext$, external. Data are averages from three experiments with S.E. $\pm$ 14%. Other experimental details are provided in the text.

![Fig. 1. Time course for mediated accumulation at 37 °C of $L$-$[^{3}H]$-5-CH$_3$-H$_4$ folate by luminal epithelial cells from mouse small intestine. A, influx by Fraction I cells at pH 6.2 and pH 7.4. B, influx by Fraction I and Fraction IV cells at pH 6.2; $ext$, external. Data are averages from three experiments with S.E. $\pm$ 14%. Other experimental details are provided in the text.](image)

Quantitative Reverse Transcription-PCR of RFC-1 cDNA—Total RNA was prepared by two treatments with RNA STAT-60 according to the manufacturer’s (TEL-TEST, Inc.) instructions. A 5-μg aliquot of methylmercuric hydroxide-treated RNA was used to synthesize cDNA by a standard procedure (29) and an oligo(dT) primer (Life Technologies, Inc.). Quantitative PCR was carried out with AmpliTag DNA polymerase (Perkin-Elmer) in the recommended buffer, 400 pmol of RFC-1 sense (5-GAGCCTTTAGGTTAGG-3′) and antisense (5′-CAAGCACCCTCCGATAG-3′) primer, 200 μM dNTPs, and 10 μM [3P]dCTP in a total volume of 50 μl. In a parallel reaction, sense (5′-TATCGACCTGGAATGGA-3′) and antisense (5′-TTACAGGTTGTATCATGCA-3′) γ-actin primers were used. After the initial denaturation step of 5 min at 95 °C, 30 cycles of 1.5 min at 95 °C, 1 min at 60 °C, and 1.5 min at 72 °C, respectively, were carried out. After this, the reaction was extended for 10 min at 72 °C. The relative amount of product formation was determined by transblotting (26) to Nytran-Plus membrane (Schleicher and Schuell) and radioautography. The amount of RFC-1 and γ-actin cDNA included in the reaction sustained amplification in the linear range of product formation at the time the amount of each product was compared.

Other Materials and Methodology—$L$-$[^{3}H]$-5-CH$_3$-H$_4$ folate (specific activity, 20 Ci/mmol) and $[^{3}H]$N-hydroxysuccinimide ester of [3H]aminopterin were purchased from Moravek Biochemicals (City of Industry, CA). $L$-$[^{3}H]$-5-CH$_3$-H$_4$ folate was purchased from Sigma Biochemical (St. Louis, MO). All other reagents were reagent grade. Radiolabeled folate compounds were analyzed for purity ($\geq$95%) by HPLC (30) before use or repurified by HPLC (30).

RESULTS AND DISCUSSION

Kinetic Delineation between Mechanisms Mediating $L$-$[^{3}H]$-5-CH$_3$-H$_4$ Folate Influx in Luminal Epithelial Cells—The velocity of initial influx of $L$-$[^{3}H]$-5-CH$_3$-H$_4$ folate was determined with Fraction I cells suspended in transport buffer with 150 μM DIDS. At this concentration of the stilbene, influx by the moderately saturable system previously described (14) for these cells was almost completely inhibited. Also, the concentration of the folate utilized (2.5 μM) was >50-fold lower than the apparent $K_i$ determined (14) for inhibition by this folate of folate compound influx by this moderately saturable system. Data shown in Fig. 1A document rapid internalization of $L$-$[^{3}H]$-5-CH$_3$-H$_4$ folate under these conditions in a pH-dependent manner. A steady-state level of internalization was achieved within 5–10 min at 37 °C at pH 6.2 or 7.4. However, initial influx and steady-state level achieved were both 4-fold greater at pH 6.2. Under the same conditions at pH 6.2, the initial influx and steady-state level of this folate achieved in Fraction IV cells (Fig. 1B) were severalfold lower. Influx mediated by the
highly saturable, pH-dependent system in Fraction IV cells is probably even lower than would appear from these data in view of the residual influx mediated by the moderately saturable system in contaminating Fraction I cells that would be expected to be present in the Fraction IV preparation.

Data on the concentration response for initial influx at 37 °C of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) in Fraction I and IV cells is shown in Fig. 2A. At pH 6.2, initial influx exhibits complex saturation kinetics in the form of highly saturable and moderately saturable components. The highly saturable component was decreased at pH 7.4 in Fraction I cells and in Fraction IV cells at pH 6.2 or pH 7.4 (data not shown). Moreover, in the presence of 150 \( \mu \text{M} \) DIDS, only a highly saturable component of influx is observed at pH 6.2 in Fraction I cells. A double-reciprocal plot of some of the data obtained in this type of experiment carried out over a wider concentration range in the presence an absence of 150 \( \mu \text{M} \) DIDS is shown in Fig. 2B. It can be seen that the two saturable components of influx observed in the absence of DIDS (Fig. 2B) are converted to a single highly saturable component in the presence of 150 \( \mu \text{M} \) DIDS. From the data presented in Fig. 2B, an apparent \( K_m \) and \( V_{max} \) for each saturable component at pH 6.2 or pH 7.4 (not shown) in Fraction I cells could be obtained. In Fraction I cells, DIDS-insensitive influx of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) exhibits (Table I) an apparent \( K_m \) in the low micromolar range and an extremely low \( V_{max} \) compared with DIDS-sensitive influx of this folate compound. Only influx \( V_{max} \) exhibited pH dependence, with the higher value for \( V_{max} \) occurring in the acidic range. In contrast, DIDS-sensitive influx of this folate compound exhibits both a high \( K_m \) and a high \( V_{max} \) neither of which are pH dependent. Therefore, at the low micromolar range of concentration or below, influx of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) by the DIDS-insensitive system would predominate in Fraction I cells, but only at pH 6.2. While at pH 7.4 and at the high micromolar range, DIDS-sensitive influx would predominate. The data in Table I also document a much lower \( V_{max} \) for \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) influx at pH 6.2 in Fraction IV cells in the presence of DIDS.

The two systems delineated above on the basis of inhibition by DIDS and pH dependence were also distinguishable by their interaction with various folate compounds as inhibitors of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) influx. In the presence of 150 \( \mu \text{M} \) DIDS, influx of this folate by the DIDS-insensitive system was inhibited (Table II) by 5-methyl- or 5-formyl-substituted \( \text{H}_4\text{folate} \) as well as folic acid in the low micromolar range, yielding values for \( K_i \) of 2–4 \( \mu \text{M} \). Similar values for \( K_i \) were obtained with the 4-aminofolate analogues, aminopterin and methotrexate. These values are consistent with other values for \( K_i \) obtained when most of these same compounds were used as inhibitors of folic acid influx by brush-border membrane vesicles in studies by others (13). In contrast, inhibition by these same compounds of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) influx by the DIDS-sensitive system (see Table II) was more variable and much less in extent.

**Table I**

**Kinetic properties of mediated \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) influx in luminal epithelial cells of mouse small intestine**

The values shown are averages ± S.E. for influx by Fraction I or Fraction IV cells in 3–5 separate experiments. Influx by the high and low affinity systems was delineated by measurements made in the presence and absence of 150 \( \mu \text{M} \) DIDS. Other experimental details are provided in the text.

| Fraction | DIDS (150 \( \mu \text{M} \)) | pH | \( K_m \) \( \mu \text{M} \) | \( V_{max} \) pmol/min/mg protein |
|----------|-------------------------------|----|-----------------|-----------------|
| I        | +                             | 6.2 | 1.8 ± 0.4       | 0.8 ± 0.2       |
|          |                               | 7.4 | 2.3 ± 0.2       | 0.2 ± 0.05      |
|          | −                             | 6.2 | 128 ± 23        | 18 ± 5          |
|          |                               | 7.4 | 115 ± 20        | 16 ± 4          |
| IV       | +                             | 6.2 | 1.9 ± 0.3       | 0.13 ± 0.03     |
|          | −                             | 6.2 | 119 ± 18        | 19 ± 4          |

**Fig. 2. Concentration response for mediated accumulation at 37 °C of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) by Fraction I and Fraction IV cells.**

A, influx following 1 min of incubation at different concentrations of \( l,L-[\text{3H}]5\text{-CH}_3\text{-H}_4\text{folate} \) in Fraction I cells at pH 6.2 ± 150 \( \mu \text{M} \) DIDS or pH 7.4 and in Fraction IV cells at pH 6.2. B, double-reciprocal plot of data on influx at pH 6.2 ± 150 \( \mu \text{M} \) DIDS; \( ext \), external. Other experimental details are provided in the text. Data are averages from three experiments with S.E. <±16%.
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**TABLE II**

Relative effects of various folate compounds as competitive inhibitors of influx of \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} \) by Fraction I cells

Influx was measured at pH 6.2 in the presence of 150 μM DIDS or at pH 7.4. Data are averages of three separate experiments ± S.E. Additional experimental details are provided in the text.

| Permeant                        | Transport system | Non-specific<sup>a</sup> |
|---------------------------------|------------------|--------------------------|
| \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} \) | Brush-border      | 2.1 ± 0.3                |
| \( \ell,\ell\text{-}[\text{H}]\text{-5-CHO-H}_4\text{folate} \)       |                  | 1.7 ± 0.2                |
| Folic acid                      |                  | 4.1 ± 0.9                |
| Aminopterin                     |                  | 3.4 ± 0.7                |
| Methotrexate                    |                  | 2.9 ± 0.4                |

<sup>a</sup> Influx of \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} (2 \mu\text{M}) \) was measured at pH 6.2 in the presence of 150 μM DIDS.

<sup>b</sup> Influx of \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} (40 \mu\text{M}) \) was measured at pH 7.4.

![SDS-polyacrylamide gel electrophoresis (PAGE) of plasma membrane proteins from Fraction I cells after affinity labeling with NHS-\( \text{[H]}\text{-aminopterin} \).](image)

...a predominant peak within the same overall range of kDa. To associate the differential in affinity labeling of Fraction I and Fraction IV cells in the form of a 58-kDa protein with influx of \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} \) by Fraction I cells, we determined the effect of affinity labeling with NHS-aminopterin on the influx of this folate compound. The data in Fig. 4 show that within the range of 0–10 μM, affinity labeling with NHS-aminopterin effectively inhibited (IC₅₀ = 6.65 ± 0.05 μM) influx of \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} \). It was also shown (data not given) that \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate} \) and folate acid were also effective inhibitors of NHS-\( \text{[H]}\text{-aminopterin labeling of Fraction I cells.}

**Detection of RFC-1 mRNA in Luminal Epithelium from Mouse Small Intestine—**The data from a Northern blot in Fig. 5 show that a substantial level of RFC-1 mRNA transcript was found with an RFC-1 cDNA probe in this tissue compared with liver when both blots were normalized to the level of β-actin RNA transcripts in each tissue. The direct detection of RFC-1 mRNA in isolated Fraction I versus Fraction IV cells by Northern blotting was not possible because of the large number of Fraction IV cells that was required for the preparation of adequate amounts of poly(A)⁺ RNA. Because of the size of these preparations (usually 20–30 mice each), the quality of the RNA was consistently poor. Therefore, quantitative reverse transcription-PCR was used to determine the relative amount of RFC-1 mRNA in preparations of much smaller numbers of Fraction I and Fraction IV cells that were obtained from only a few mice.

The results of these reverse transcription-PCR reactions in the form of a radioautograph are given in Fig. 6. The data shown are for the amount of RFC-1 cDNA product obtained normalized to the amount of β-actin cDNA product obtained when run in a parallel PCR reaction. From Fig. 6, it can be seen that RFC-1 mRNA is present in Fraction IV cells at a much lower level than in Fraction I cells.

**Western Blotting—**Further evidence for an association between \( \ell,\ell\text{-}[\text{H}]\text{-5-CH}_3\text{-H}_4\text{folate influx} \) in Fraction I cells and RFC-1 gene expression was obtained in the form of immunoblotting with antibodies raised in rabbits against anti-RFC-1-specific peptides. The blots in Fig. 7 reveal a prominent protein band at 58 kDa and a faint band at 46 kDa in plasma membrane from Fraction I cells. In contrast, blotting of plasma membrane from Fraction IV cells revealed neither of these protein bands but occasionally detected a weakly reacting protein band in the vicinity of 35 kDa. Western blotting with anti-RFC-1 peptide antibody was also carried out with purified brush-border membrane protein from Fraction I cells. From data shown in Fig. 6B, it can be seen that this antibody detected a 58-kDa protein band in this specialized membrane in addition to another band at ~35 kDa. A plot of plasma membrane protein from Fraction IV cells run in parallel is also...
The results of two representative experiments. 

Additional experimental details are given in the text. Data are representative results from one of several blots.

Further Evidence for a Role of the Transporter Encoded by the RFC-1 Gene in Folate Absorption—The results of both NHS-[3H]aminopterin affinity labeling and immunoblotting with anti-RFC-1 peptide antibody presented above clearly suggest that there is an association between a 58-kDa protein and folate transport in absorptive luminal epithelium (Fraction I cells) from mouse small intestine. More direct evidence was obtained by determining the effect of the anti-RFC-1 peptide antibody on L-L-[3H]-5-CH₃-H₄folate influx by Fraction I cells at pH 6.2 in the presence of 150 μM DIDS. The results presented in Fig. 8 show that influx of this folate compound under these conditions by Fraction I cells was inhibited substantially by the prior incubation of Fraction I cells with varying amounts of anti-RFC-1 peptide antibody and that inhibition occurred in a concentration-dependent manner. No effect was obtained with an irrelevant antibody of the same IgG class against a mouse folylpolyglutamate synthetase peptide purified in the same manner from rabbit serum.

The studies described here on folate compound influx by isolated luminal epithelial cells from mouse small intestine are in close agreement with those published earlier (11, 13) by Selhub and Rosenberg using either everted sacs or brush-border membrane vesicles from rat small intestine. Under conditions that suppress non-pH-dependent influx (14), the role of a highly saturable, pH-dependent system in mediating influx of folate compounds that operates only in absorptive epithelial cells has been documented with characteristics very similar to that found (13) in the brush-border membrane of rat small intestine. These studies were further extended to show that the dependence of influx of L-L-[3H]-5-CH₃-H₄folate on pH reflected a difference at each pH in values for Vmax but not for apparent Ka for this system. The basis for this effect on maximum capacity rather than saturability is not understood but could reflect a difference in the rate of translocation of the transporter in response to a difference in the proton gradient (inside versus outside) at each pH.

In further support of the notion that the acid pH-dependent system in the luminal epithelium of mouse small intestine is involved in folate absorption, we have also provided data that show that acid pH-dependent transport in luminal epithelium of this organ is developmentally regulated. Physiologically significant levels of acid pH-dependent influx of L-L-[3H]-5-CH₃-H₄folate and of the 58-kDa putative transporter occurs only in mature absorptive cells apparently within the brush-border membrane. The relevance of this protein to acid pH-dependent transport in these cells was strongly suggested by the results of folate-based affinity labeling and immunoblotting. Also, the murine RFC-1 gene, which encodes a 58-kDa membrane protein (15), appears primarily to be transcriptionally active in mature absorptive cells. Finally, it was found that the folate-based affinity label and antibodies against murine RFC-1-specific peptides will inhibit influx of L-L-[3H]-5-CH₃-H₄folate in mature absorptive cells.

In the report by Said et al. (16), the RFC-1 cDNA derived from poly(A)⁺ RNA of mouse small intestine incorporated a different 5’ end from that initially reported for an RFC-1 cDNA derived from L1210 cell poly(A)⁺ RNA. We have now obtained data to show (31) that the RFC-1 cDNA derived from mouse small intestine is a splice variant incorporating an alternative to exon 1 of the RFC-1 gene, which encodes this different 5’ end. Although supporting data are still needed, it is likely that transcription of this splice variant, which is initiated at the alternate to exon 1 in small intestine, is under the control of a second promoter. Transcription of genes that encode alternate 5’ ends in the form of splice variants have frequently been shown (32–35) to be under the control of multiple promoters. Additional work will be required to establish this point.

Lastly, it is of interest to compare data obtained in the current studies on mediated folate compound transport in luminal epithelium of small intestine to that documented (1, 2, 12, 20, 36) in detail for L1210 cells. If we assume that transport of these compounds in both of these tissues is a direct result of RFC-1 gene expression alone, a paradox emerges pertaining to the properties of the transport system in each case. Although the murine RFC-1 gene encodes a 58-kDa protein, the mass of this protein actually detected is 58 kDa in luminal intestinal epithelium but only 46 kDa in L1210 cells (20, 36). Moreover, discrepancies exist with regard to both pH dependence and structural preferences among folate compounds as permeants. The transport system in L1210 cells exhibits (12) a moderately alkaline pH optimum and marked differences (1, 2, 12, 18) in preferences among folate compounds as permeants. In contrast, the system in mouse luminal intestinal epithelium ex-
hibits (see above) an acid pH optimum and close similarity in preferences among the same folate compounds as permeants. There are a number of possible explanations for these apparent discrepancies that are consistent with the role of the RFC-1 gene in each case. These could pertain to alternate splicing, post-transcriptional modification and (or) the role of accessory proteins. However, since there is currently a lack of relevant data, further work will be required that addresses each aspect of this question.

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