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Complementation of a Methotrexate Uptake Defect in Chinese Hamster Ovary Cells by DNA-Mediated Gene Transfer

T. MICHAEL UNDERHILL AND WAYNE F. FLINTOFF*

Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1

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A methotrexate-resistant Chinese hamster ovary cell line deficient in methotrexate uptake has been complemented to methotrexate sensitivity by transfection with DNA isolated from either wild-type Chinese hamster ovary or human G2 cells. Primary and secondary transfectants regained the ability to take up methotrexate in a manner similar to that of wild-type cells, and in the case of those transfected with human DNA, to contain human-specific DNA sequences. The complementation by DNA-mediated gene transfer of this methotrexate-resistant phenotype provides a basis for the cloning of a gene involved in methotrexate uptake.

The folic acid analog methotrexate (Mtx) is a cytotoxic agent to dividing mammalian cells which exerts its effect by inhibiting the enzyme dihydrofolate reductase (2). To achieve this function, the drug must be transported across the cell membrane and released intracellularly. It is believed that this transport process is mediated by an energy-dependent carrier system requiring anion exchange (14). There is conflicting evidence from various studies as to whether the analog is transported by the same system that allows folic acid to enter the cell or whether a separate system for reduced folate is employed. Kinetic and competition analyses in L1210 mouse cells have shown that two systems exist under one set of conditions (26, 31), while only one system exists under alternative conditions (13). In addition, some mutants are defective in uptake of both Mtx and folic acid, while others are defective in uptake of only Mtx (9, 10; T. M. Underhill and W. F. Flintoff, Somat. Cell Mol. Genet., in press). Also, some Mtx-deficient mutants have increased requirements for reduced folates to maintain growth without a concomitant increase in the requirement for folic acid (18, 25, 27). Taken together, these observations suggest that some but not all components may be shared in the uptake of these two compounds. In addition, some Mtx- and folate-binding proteins present in cell membranes have been identified (1, 5, 6, 12, 15, 16, 19, 20, 22), although their role in the overall uptake process is unclear. A recent report, however, indicates that a mouse membrane 45- to 48-kilodalton protein may be involved (32).

In a recent report, we have described an Mtx-resistant Chinese hamster ovary (CHO) cell line, designated Mtx RII Oua^R 2-4, that was unable to transport Mtx but was able to take up folic acid in a manner similar to that of wild-type cells (Underhill and Flintoff, in press). Under conditions of folic acid-free medium, this cell line required 250 times the level of folinic acid required by wild-type cells to maintain optimum growth. This requirement for high levels of folinic acid in otherwise folic acid-free medium is a common property shown by several mutants defective in Mtx uptake (17, 27). Pseudorevertants selected from this cell line for growth on low levels of folinic acid retained their resistance to Mtx. Because the Mtx-resistant phenotype was a genetically recessive trait (8), it should be possible to complement this phenotype by transfection with DNA from an Mtx-sensitive or wild-type cell. Such an approach of DNA-mediated gene transfer has been previously used to complement several recessive mutations and to identify genes that encode proteins that either represent a small fraction of total cellular protein or are difficult to isolate (18, 23, 24, 29, 30). In this report we demonstrate that it was possible to isolate cells that had regained their sensitivity to Mtx after transfection of the Mtx RII Oua^R 2-4 cell line with genomic DNA from either wild-type CHO or human G2 cells.

MATERIALS AND METHODS

Cells. The source and routine propagation of the wild-type and mutant CHO Mtx RII Oua^R 2-4 cells have been previously described (7, 8). The human G2 cells (21) were obtained from G. Mackie and were maintained as monolayer cultures under conditions similar to those used for the CHO cells.

DNA isolation. High-molecular-weight DNA was isolated from exponentially growing cells by the procedure of Gross-Bellard et al. (11).

Transfection. Transfection of DNA into recipient Mtx RII Oua^R 2-4 cells was carried out by the Polybrene procedure described by Chaney et al. (4). Briefly, 5×10^5 Mtx RII Oua^R cells per T-75 flask were treated for 6 h with 3 ml of alpha medium supplemented with 10% fetal bovine serum, 10 to 60 μg of DNA sheared by passage three times through a 21-gauge needle to an approximate size of 40 to 50 kilobases, and 30 μg of Polybrene (Aldrich Chemical Co., Inc.). Subsequently, the DNA mixture was removed and replaced with 5 ml of alpha medium containing 10% fetal bovine serum and 30% dimethyl sulfoxide for 4 min. The solution was removed, and the cells were washed in alpha medium containing fetal bovine serum and allowed to recover for 24 h. The cells were washed with phosphate-buffered saline and fed alpha medium deficient in folic acid and supplemented with 10% dialyzed fetal bovine serum and 2×10^{-9} M folinic acid. Individual colonies were picked 2 to 3 weeks later and subjected to further analysis. Transfectants generated with wild-type CHO DNA were given the designations T1, T2, etc., and those generated with human DNA were designated HT1, HT2, etc. Secondary transfectants generated with DNA from the Mtx-sensitive HT2 cell line were given the designations HT2-1, HT2-2, etc.

Southern blotting. High-molecular-weight DNA was digested with a sixfold excess of *Eco*RI (Pharmacia, Inc.) according to the conditions recommended by the supplier, separated on a 0.8% agarose gel, and transferred onto

* Corresponding author.

nitrocellulose paper by the method of Southern (28). Blots were prehybridized for 24 h at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution–50 μg of salmon sperm DNA per ml–300 μg of yeast tRNA per ml–0.1% sodium dodecyl sulfate–50 mM sodium phosphate (pH 6.5)–10% dextran sulfate. Sheared high-molecular-weight human DNA was nick translated with [^{32}P]dCTP (ICN Pharmaceuticals, Inc.; 3,200 Ci/mmol) and [^{32}P]dGTP (ICN; 3,200 Ci/mmol) by using the Bethesda Research Laboratories nick translation kit to a specific activity of 5×10^8 cpm/μg. DNA was added to the prehybridization cocktail to a final concentration of 3.8 ng/ml. After 48 h at 42°C, blots were washed twice with 2× SSC–0.1% sodium dodecyl sulfate at 23°C and 3 times with 0.1× SSC–0.1% sodium dodecyl sulfate at 58°C and rinsed with 0.1× SSC–0.1% sodium dodecyl sulfate and 0.1× SSC and exposed at −70°C to an X-ray film with Cronex Lightning-Plus intensifying screens.

Phenotype testing. The resistances of various cell lines to Mtx were determined by dose-response curves as previously described (7) and expressed as D_{10} values, the drug concentrations that reduced cell survival to 10%.

Drug uptake and kinetic analysis. The uptake of 0.25 μM of ^{3}H-labeled Mtx into various cell lines was determined as previously described (Underhill and Flintoff, in press) except that uptake was performed on monolayer cultures rather than in suspension. The uptake was normalized to picomoles per milligram of protein. Protein content was determined by the method of Bradford (3) with the BioRad Protein Microassay.

Kinetic analyses of uptake employed the same method as described above, except assays were designed to contain various levels of labeled drug with various specific activities. Samples were taken in the linear range of uptake at three time points and graphed by double reciprocal plots, and the kinetic parameters were determined.

RESULTS AND DISCUSSION

The observation that Mtx-resistant transport-deficient cells required 100 to 250 times the wild-type level of folinic acid in otherwise folic acid-deficient medium to maintain optimum growth provided an opportunity to use this as a potential reversion scheme for the Mtx-resistant phenotype (Underhill and Flintoff, in press). Lines selected from some Mtx RII mutants that grew on low levels of folinic acid were also reverted to Mtx sensitivity. The frequency of reversion for growth on low levels of folinic acid and to Mtx sensitivity was approximately 10^{-4} (Underhill and Flintoff, in press). In another case, pseudorevertants from Mtx RII Oua^R 2-4 that were able to grow on low levels of folinic acid but retained their resistance to Mtx were obtained. The frequency for reversion for growth on low levels of folinic acid was 10^{-6}, but reversion back to Mtx sensitivity was $<10^{-7}$. The low reversion of the Mtx-resistant phenotype in the Mtx RII Oua^R 2-4 cells under these selective conditions raised the possibility of using this line and these conditions to complement the Mtx-resistant phenotype by transfer of DNA from wild-type or Mtx-sensitive cells. Surviving colonies could be easily tested to determine whether a transfection event or a

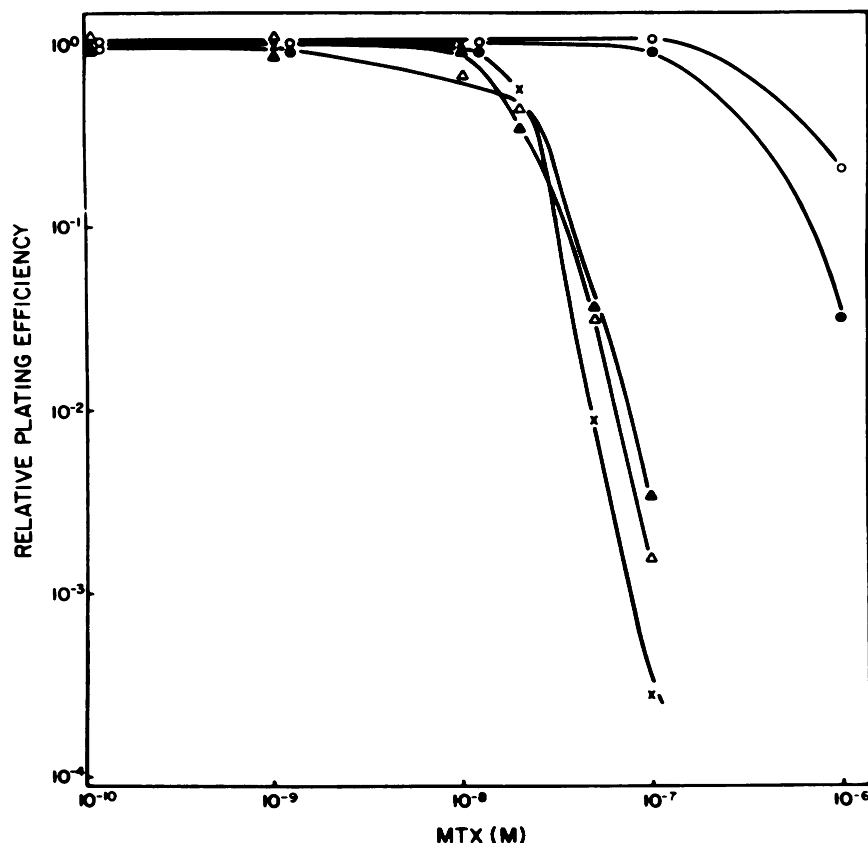

FIG. 1. Dose-response curves for cells growing in Mtx. Exponentially growing cells were plated in selective medium with various concentrations of Mtx. ×, Wild type; ○, Mtx RII Oua^R 2-4 mutant; ●, isolate HT13 generated with human DNA; ▲, transfectant HT7 generated with human DNA; △, transfectant T1 generated with wild-type CHO DNA.

TABLE 1. Transfection frequencies[a]

DNA source	Colonies	No. of colonies tested	No. of Mtx[s] colonies[b]	Frequency of Mtx[s] (colonies/30 μg of DNA per 10^6 cells plated)
No DNA	31	10	0	
Primary transfection				
Wild-type CHO	4	4	2	0.8
Wild-type CHO	5	5	4	1.6
Human G2	16	13	8	0.8
Mtx RII Oua[R] 2-4	9	9	0	<0.03
Secondary transfection				
HT2-2	8	8	6	0.8
HT2-7	5	5	4	0.5

[a] Mtx RII Oua[R] 2-4 cells were transfected as described in Materials and Methods.

[b] Mtx[s] isolates were defined as having Mtx sensitivities similar to that of wild-type CHO cells with a D_{10} of 2×10^{-8} M.

reversion event had occurred, since the former should yield isolates with an Mtx-sensitive phenotype and the latter should yield isolates with an Mtx-resistant phenotype.

Mtx RII Oua[R] 2-4 cells were transfected with sheared DNA from either wild-type CHO or human G2 cells. As shown in Table 1 and Fig. 1, about 65% of all the tested primary isolates surviving these selective conditions had regained their sensitivity to Mtx. The remaining 35% retained the parental phenotype of Mtx resistance and most likely represented those that have reverted for folinic acid requirement, as this frequency was comparable with the spontaneous reversion frequency of 10^{-6}. In a similar experiment, in which these cells were transfected with DNA isolated from the Mtx RII Oua[R] 2-4 cells, no Mtx-sensitive isolates were obtained; all isolates examined that survived under low folinic acid growth were Mtx resistant.

To confirm that the Mtx-sensitive isolates generated by this primary transfection with human DNA represented true transfectants, DNA was isolated from several of these lines and tested for the presence of human-specific DNA sequences by hybridization with a radiolabeled total human DNA as probe. Figure 2 shows an autoradiogram of three Mtx-sensitive primary isolates obtained with human DNA as donor. All these lines contained a human-specific DNA fragment of about 9.5 kilobases in size with diffuse signal spread throughout each lane. DNA from the recipient CHO cells showed no hybridization with the human DNA as probe under these conditions. One isolate that was Mtx-sensitive and was obtained after transfection with human DNA showed no significant hybridization with the human probe (data not shown). The ability to detect human sequences in these transfectants was a function of the amount of human DNA taken up during transfection and its subsequent stabilization and incorporation into the recipient cellular genome. The fact that not all Mtx-sensitive isolates examined appeared to contain human-specific sequences does not discount the possibility that these sequences could be present in low abundance and hence be undetectable. A similar variability in the amount and detection of human DNA sequences in transfected systems with human DNA has been reported by others (23, 24, 29). It should be mentioned that there is a band of very low intensity in the lane containing DNA from a supposed Mtx-resistant isolate (Fig. 2, lane B); upon further testing it was found that this isolate was partially resistant to Mtx and was able to transport a limited amount of Mtx relative to wild-type CHO cells (unpublished

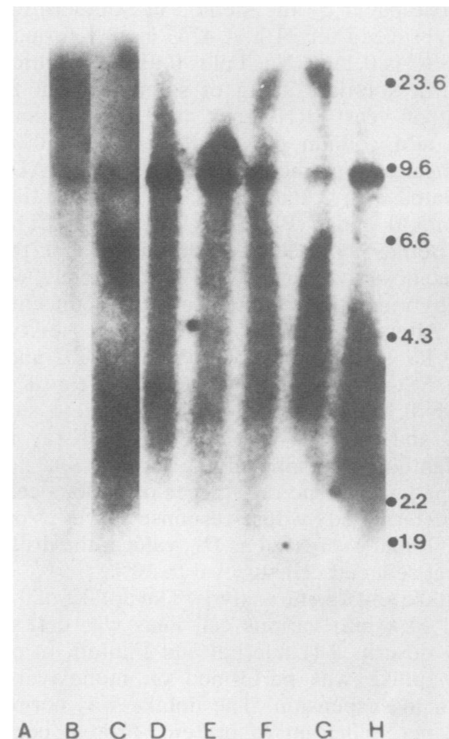

FIG. 2. Autoradiograms of Southern hybridizations of wild-type CHO and human DNA-generated transfectant DNAs and total nick-translated ^{32}P-labeled human DNA. DNA (15 μg) from each transfectant line and from the CHO line Mtx RII Oua[R] 2-4 was digested with *Eco*RI and subjected to electrophoresis in a 0.8% agarose gel. Lanes: A, wild-type CHO; B, Mtx-resistant isolate HT13 generated with human DNA; C, D, and E, Mtx-sensitive transfectants HT4, HT2, and HT1, generated with human DNA, respectively; F, G, and H, Mtx-sensitive transfectants HT2-1, HT2-2, and HT2-3 generated with DNA from HT2, respectively. ●, Positions of molecular weight markers generated by digestion of λ phage DNA with *Hin*dIII. (Lanes A and D through H are at the same exposure; lanes B and C are at a longer exposure to bring out the hybridization signal.)

data). Thus, there was a strong correlation with the presence of human DNA sequences and the Mtx-sensitive phenotype in these primary transfectants generated using human DNA as the donor. On the basis of the phenotypic properties of the recipient cell line, it seems reasonable to conclude that the isolates obtained by using DNA from the wild-type CHO cells as the donor also represented true transfectants.

Secondary transfectants were generated to determine whether a similar pattern of human DNA sequences were retained. DNA was isolated from two Mtx-sensitive primary transfectants and used to transfect the Mtx RII Oua[R] 2-4 cells by the method described above. About 75% of the total number of secondary transfectants obtained were Mtx sensitive (Table 1). DNA from three of these (HT2-1, HT2-2, and HT2-3) were analyzed for the presence of human-specific DNA sequences. As shown in Fig. 2, the hybridization pattern in the secondary transfectants was similar to that of the primary ones. The presence of a similar human-specific DNA fragment in several independently selected primary and secondary transfectants suggests that the function conferring Mtx sensitivity on these transfectants may be linked to this fragment of DNA.

If these transfectants did in fact represent complementation of the Mtx-resistant phenotype, then they should have

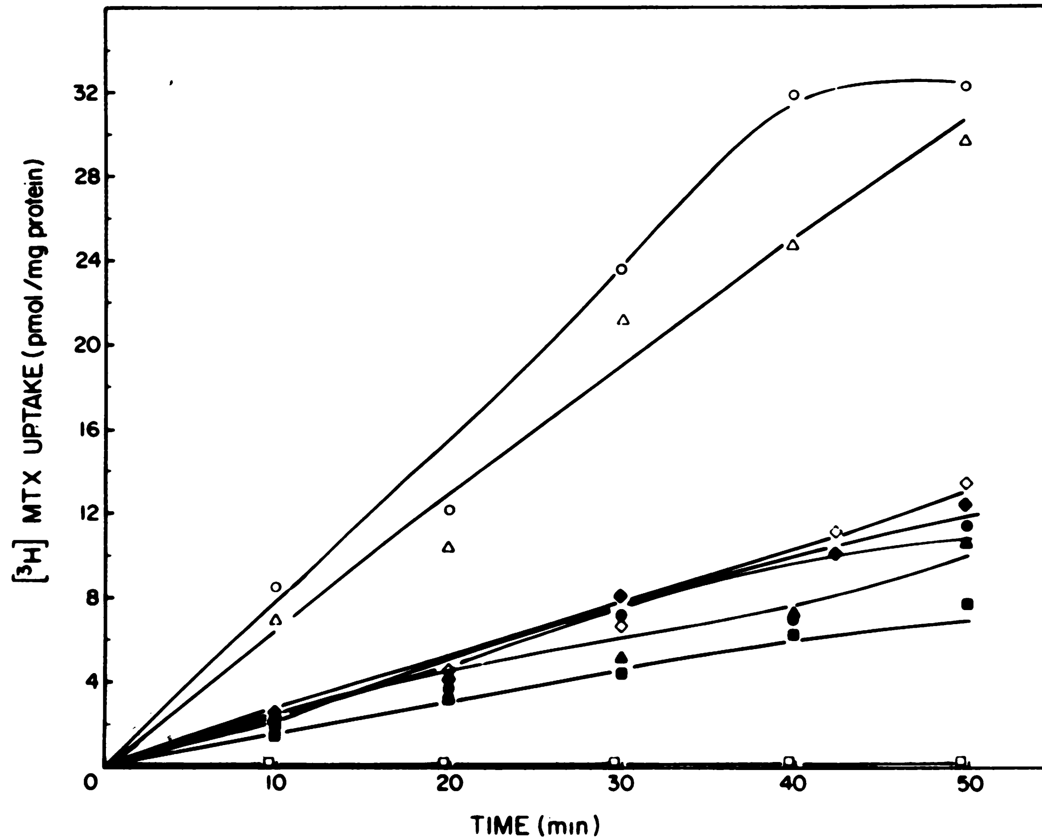

FIG. 3. Cellular uptake of 0.25 μM ^{3}H-labeled Mtx. Cells were incubated at 37°C, and [^{3}H]Mtx uptake was measured as described in Materials and Methods. △, Wild-type; □, Mtx RII Oua^R 2-4; ●, transfectant T1 generated with wild-type CHO DNA; ▲, transfectant T11 generated with wild-type CHO DNA; ■, transfectant HT2 generated with human DNA; ○, transfectant HT7 generated with human DNA; ◇, secondary transfectant HT2-1; ◆, secondary transfectant HT2-2.

regained the ability to take up Mtx in a manner similar to that of wild-type cells. Representative primary and secondary transfectants accumulated labeled Mtx, albeit not to the same level as wild-type cells (Fig. 3). This is consistent with their Mtx-sensitive phenotype. The K_T associated with the newly acquired Mtx uptake in the Mtx-sensitive transfectants was not unlike that of wild-type CHO or human G2 cells, which had K_Ts of approximately 0.6 μM. Since the K_T values for Mtx uptake in both the parental human G2 and wild-type CHO cells were similar, it was not possible to functionally distinguish between wild-type- or human DNA-generated transfectants. In some cases, the transfectants had a reduced V_{max} for Mtx uptake compared with wild-type cells (data not shown). Because transfected DNA may integrate at random sites, perhaps this is a consequence of association in the genome of the recipient cell in which expression was reduced.

The availability of an Mtx-resistant line with a defect in drug uptake that showed no detectable reversion to Mtx sensitivity under low-folate growth conditions has provided a unique opportunity to use DNA-mediated gene transfer to complement this Mtx-resistant phenotype. Isolates obtained by using either wild-type CHO or human cells as a source of donor DNA regained the sensitivity to Mtx, the ability to take up Mtx, and, in the case of human donor DNA, the presence of human-specific DNA sequences.

This ability to complement the Mtx-resistant phenotype in the Mtx RII Oua^R 2-4 cell line by DNA-mediated transfer and to regain the ability to transport Mtx is, to our knowledge, the first example of such an observation for this phenotype. At present it is not known what the nature of the alteration that confers resistance to the drug is in this cell line. The ability to revert these cells to Mtx sensitivity by DNA-mediated gene transfer coupled with the outlined selection scheme provides an opportunity to design strategies to clone the gene responsible. The availability of such a gene should allow identification of the mutational basis for the resistant phenotype and should be useful in the molecular analysis of the Mtx uptake system. Such studies are in progress.

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