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Organization of the Methotrexate Resistant Mouse L5178YR Dihydrofolate Reductase Gene and Transformation of Human HCT-8 Cells by This Gene

D. I. Scheer, S. Srimatkandada, B. A. Kamen, S. Dube, and J. R. Bertino

A. Introduction

Gene amplification has been recently shown to play a pivotal role in the development of resistance to the folate analogue, methotrexate (MTX), in several cell culture systems (Schimke et al. 1978). Stepwise increases in the concentration of MTX supplied to the medium in which the murine lymphoblastoid line L5178Y was cultivated resulted in the development of 100,000-fold elevation in resistance (Dolnick et al. 1979). The resistant line, L5178YR, was able to grow in the presence of millimolar concentrations of MTX, and it was shown that these cells had a 300-fold elevation in the activity of the target enzyme, dihydrofolate reductase (DHFR). Moreover, the increase in DHFR activity could be correlated with a proportional increase in DHFR-specific mRNA (300-fold) as well as a 300 fold-increase in DHFR gene copies.

To clarify the mechanism of the amplification event as well as gain insight into the regulation of the DHFR gene an analysis has been made of the DNA sequence organization of the DHFR gene. Further, DNA-mediated gene transfer has been employed to transform a human colon carcinoma line (HCT-8) with DHFR genes from the L5178YR line.

B. Methods and Materials

I. Cell Culture

Mouse L5178Y and L5178YR lines were maintained in Fischer's medium with 10% horse serum. Human colon carcinoma, HCT-8, was maintained in RPMI medium containing 20% fetal calf serum.

II. Isolation of DNAs

Essentially as described by Blin and Stafford (1976)

III. Transformation

Essentially as described by Graham and van der Eb (1973) with minor modifications.

IV. Enzymology

Essentially as described by Hayman et al (1978) for DHFR and by Lomax and Greenberg (1967) for TMP synthetase, with modifications by Dolnick and Cheng (1977) and Kamen et al. (1976) for the cofactor binding studies.

V. Southern Blot Hybridization

Southern blot hybridization was done essentially as described by Southern (1975) with modifications by Wahl et al. (1979). Cloned DHFR cDNA probes were kindly provided by Dr. R. Schimke (See Chang et al. 1978).

C. Results and Discussion

Data obtained from our laboratory as well as that of Schimke (Nunberg et al. 1980) suggests that the murine DHFR gene is interrupted by at least five intervening sequences. Figure 1 depicts a Southern blot of amplified L5178YR DNA which has been cleaved by several enzymes, transferred to DBM paper, and hybridized with the cloned DHFR cDNA probes (pDHFR 21 and 26). Since none of the indicated enzymes cleave within the structural gene, the patterns of fragmentation result from sites provided by the intervening sequences. From limited and complete Pst1 digestion

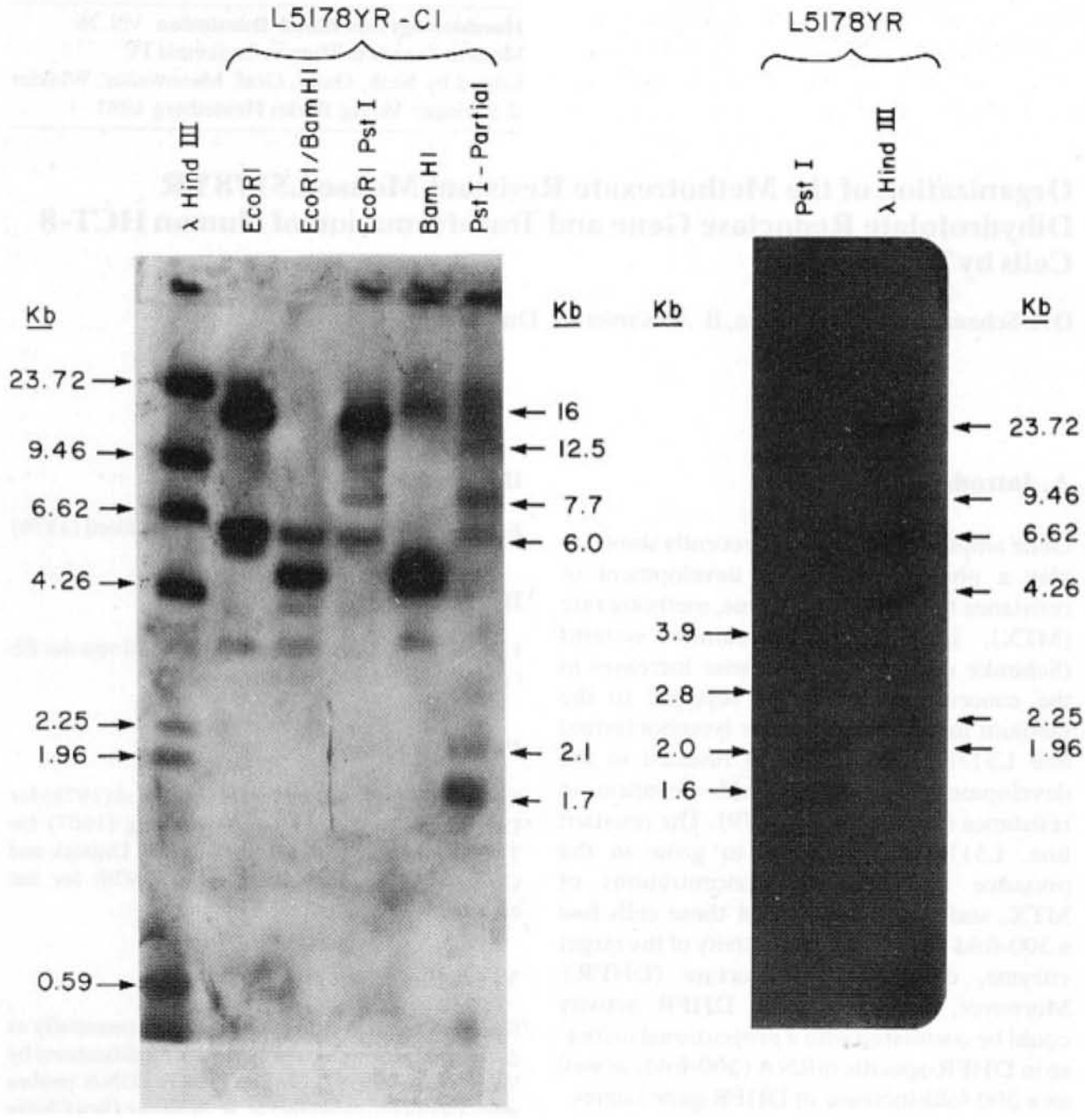

Fig. 1. Southern blot hybridization of L5178YR DNA. *Left* DNA was cleaved with the indicated restriction endonucleases *(Eco R1)* and electrophoresed on 0.8% Agarose, transferred to DBM paper, and hybridized with ^{32}P-labelled pDHFR 21 cDNA probe. *Right* Similar conditions as above except the hybridization was carried with ^{32}P-labelled pDHFR 26 DNA probe. λDNA cleaved with HindIII and labelled with ^{32}P was used as molecular weight marker. *Kb*, kilo base

intervening sequence structure as well as gene size may be deduced. A minimum size estimate for the gene is 45 kilobase pairs. This figure agrees with the estimate of Nunberg et al. (1980) that the DHFR gene is at least 42 Kb in size.

Transformation of the human colon carcinoma (HCT-8) line was obtained with L5178YR DNA as a $CaPO_4$ precipitate. Selection for transformants was carried out at concentrations of MTX ranging from $10^{-7}M$ to $10^{-3}M$. Two transformant lines remain stably resistant after approximately 12 months, one at $10^{-7}M$ and a second at $10^{-6}M$ (C_1HCT 8-6). C_1HCT 8-6 was analyzed for levels of DHFR enzyme activity and found to contain a ten fold elevation compared to parental HCT-8. Further, in examining other enzymes in the folate pathway it was found that TMP-synthetase was also elevated in this line by five fold.

To determine the species of the elevated enzyme in C_1HCT 8-6 cofactor binding studies

were carried out with ^{3}H-MTX in the presence of excess NADPH. Under the conditions of assay C_1HCT 8-6 enzyme exhibits MTX binding that closely approximates the kinetics expected for mouse (L5178YR C3 enzyme), whereas the HCT-8 enzyme binds MTX with a K_D at least 100-fold higher than L5178YR C3 enzyme (data not shown).

Southern blot hybridization analysis of the human DHFR gene and the transformed DHFR gene is depicted in Fig. 2. Results indicate a qualitative as well as a quantitative difference in the sequences corresponding to the DHFR gene from the two sources. That the C_1HCT 8-6 pattern corresponds to mouse is supported by the following observations. The cell line exhibits a stably resistant phenotype in the absence of drug selection for a period of 6 months. Thus, the information has most likely become associated with the recipient genome. And, assuming an integrative recombination event as depicted in Fig. 3, a truncation from the 3′ terminal fragment (EcoR1 16 kb) consisting of approximately 10 kb would result in the observed restriction pattern that is, the 6.1 kb, 5.8 kb, and a ca. 6 kb fragment. The presence of a minor higher molecular weight (14 kb) fragment in the C_1HCT 8-6, which is absent from the human gene, is a further argument for mouse information in the transformed line. Since the line has not been cloned, it is possible that a heterogeneous cell population could account for isolated integrative events that correspond to less sequence loss at the 3′ end of the gene. Thus, a 14 kb fragment could result from a 2 kb truncation from the 16 kb fragment. At the 5′ end earlier data indicated the presence of a 2.3

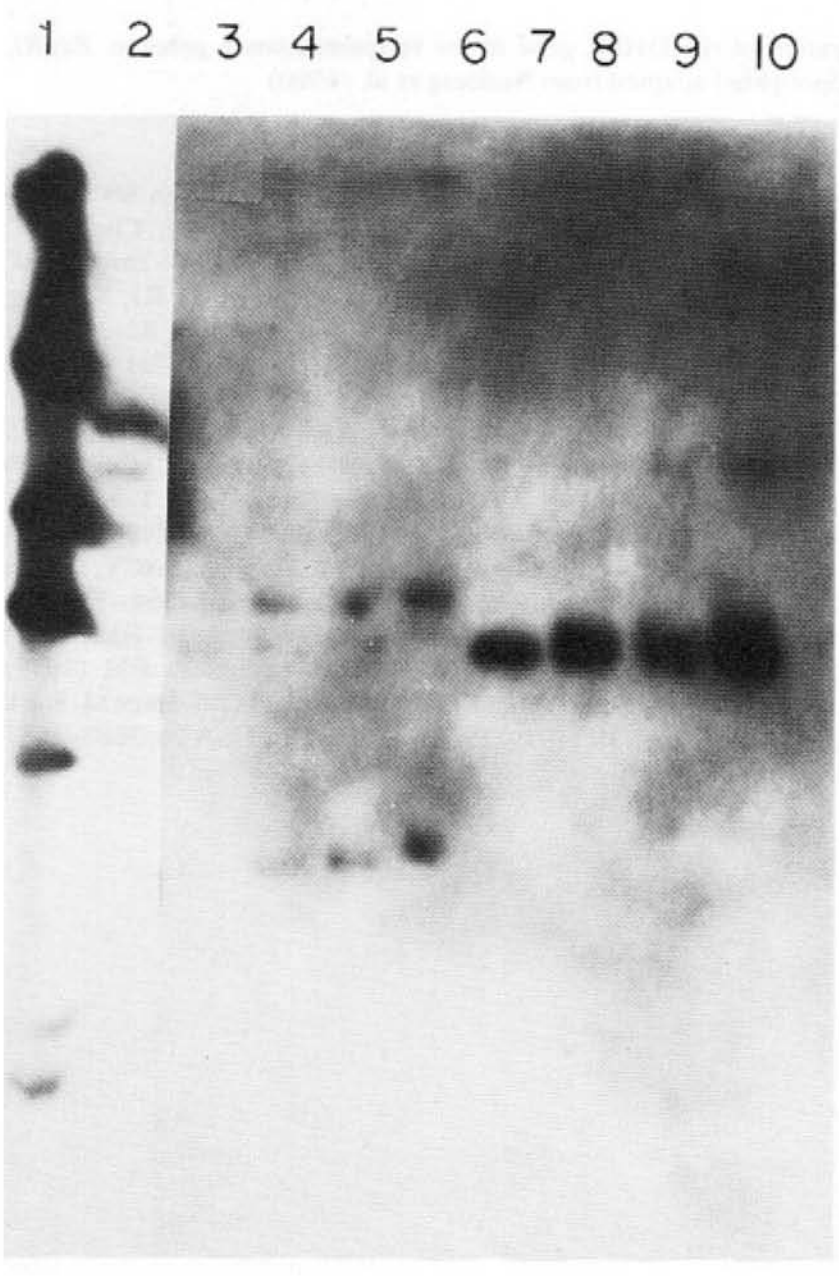

Fig. 2. Southern blot hybridization of human placental DNA (lanes 3 to 6) and C_1HCT8-6 DNA (lanes 7 to 10). Restriction endonuclease *(EcoR1)* was employed for both DNAs. Conditions were otherwise similar to those of Fig. 1. Lanes 3 to 6 and lanes 7 through 10 demonstrate hybridization with increasing concentrations (e.g., 2.5 mcg. to 10 mcg.) for each DNA

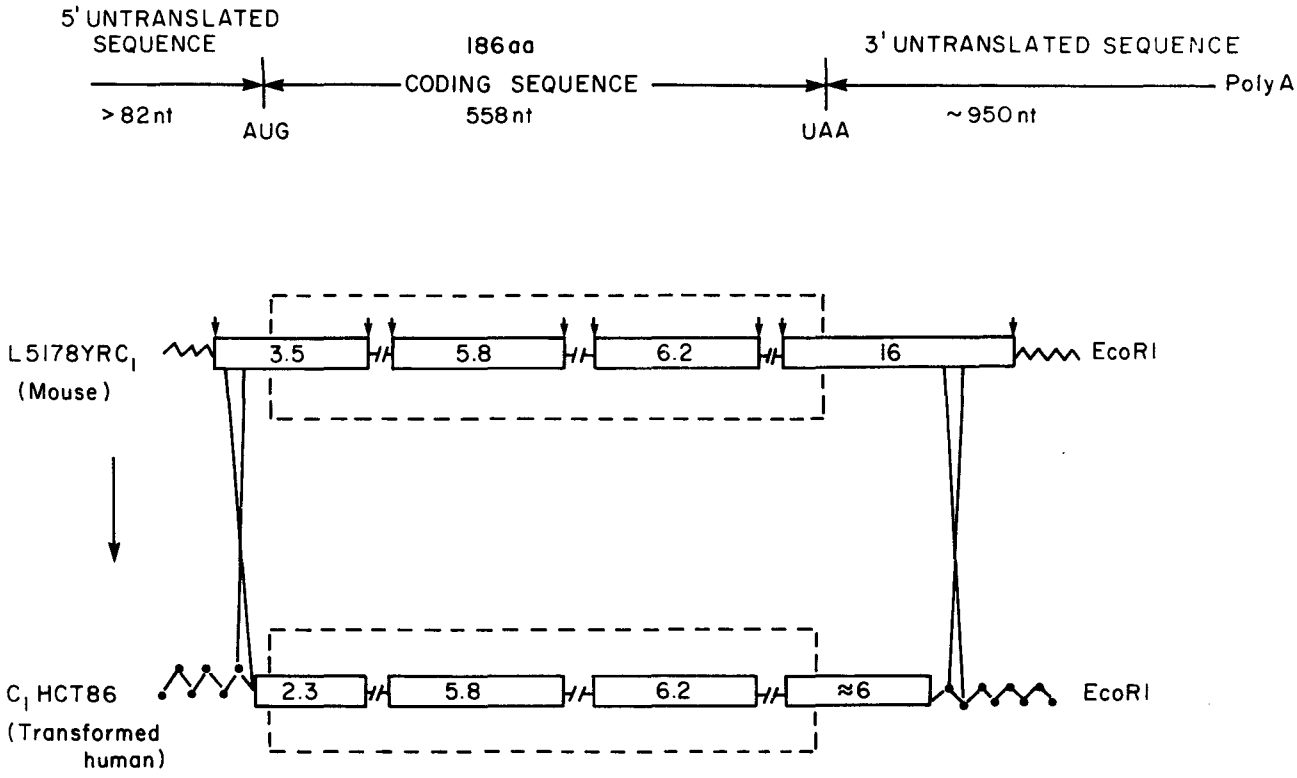

Fig. 3. Proposed model for the integration of the DHFR gene in the recipient human genome. *EcoRI*, restriction endonuclease. Data from upper panel adapted from Nunberg et al. (1980)

kb fragment which might result from a truncation of the 3.5 kb fragment during integration. However, we are trying to resolve whether such is the case in the light of more recent data where the 5′ terminal fragment appears to be 3.5 kb. Definitive structural data on the transformed sequence awaits the characterization of fragments of the gene obtained by molecular cloning.

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