Quantitative evaluation of intracellular sense: antisense RNA hybrid duplexes

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ABSTRACT

Previous studies have demonstrated that for an antisense RNA to be effective in attenuating gene expression, a large but indeterminate excess of antisense RNA is required. To quantitatively evaluate RNA hybrid duplex formation, expression vectors containing antisense dihydrofolate reductase (DHFR) cDNAs were transfected into KB and KB-1BT (a DHFR overexpressing variant) cells and transfectants expressing antisense transcripts of exon 1 through intron 1 (ex1-I) or exons 1 through 4 (ex1-4) were analyzed for hybrid duplex formation. Stable duplexes were detectable in KB-1BT but not in KB cells. Approximately 5–9% of antisense ex1-I RNA and 20–37% of antisense ex1-4 RNA were found in duplexes. The amount of each hybrid duplex RNA was found to be a linear function of intracellular single-stranded antisense RNA levels and a hybrid index, $H_{s:as}$, was devised to describe this relationship. Based upon the value of $H_{s:as}$ for each antisense RNA:mRNA duplex, it is calculated that an approximate 2,800- and 600-fold excess of ex1-I and ex1-4 antisense RNA are respectively required for 50% of DHFR mRNA to be present in duplexes. Results support the hypothesis that intracellular sense:antisense RNA hybrid duplex formation is inefficient and dependent upon the levels, lengths and possibly the structures of the RNAs involved.

INTRODUCTION

Antisense nucleic acids have attracted much interest as possible means to modulate gene expression (1–4). However, many questions related to their mechanism, efficiency, specificity and toxicity still remain. The presumed critical step responsible for putative antisense effects is the interaction of antisense nucleic acids with target RNAs. The formation of hybrid duplexes between sense and antisense molecules is thought to be a prerequisite for the suppression of targeted gene function (5–7).

To date, several groups have reported the detection of duplexes formed between cellular mRNA and antisense RNAs in mammalian cells. Kim and Wold (5) reported that in mouse L

cells transfected to express an antisense herpes simplex virus thymidine kinase (HSV-TK) RNA, most HSV-TK RNA was found in duplex form when the ratio of antisense to sense HSV-TK RNA was approximately 300. In these cells TK activity was reduced 80–90%. Yokoyama and Imamoto detected duplexes of sense and antisense myc RNAs when intracellular antisense RNA was present at 10–20 fold that of myc mRNA (6). Krystal *et al.* (8) detected cytoplasmic duplex formation between N-myc mRNA and an endogenous antisense transcript. Detection of duplex formation, presumably from interactions of sense RNA and antisense nucleic acids, has been reported in other cell systems as well (7, 9, 10).

Even though there is a general consensus, supported by the above mentioned reports, that the formation of intracellular sense:antisense hybrid duplex is the molecular basis for antisense effects and that a large excess of antisense to sense RNA may be needed, there is a lack of information of the quantitative relationship of hybrid duplexes with the levels and structures of sense and antisense RNAs.

In this study, we report studies of a cell culture model for the quantitative evaluation of intracellular sense:antisense RNA hybrid duplex formation. Human dihydrofolate reductase (DHFR) mRNA was targeted by antisense RNAs produced from episomal vectors. DHFR mRNA was targeted since the DHFR gene, RNA and protein have been well characterized (11–15) and because extensive depression of DHFR enzyme activity is required before any cytotoxic effects become manifest. This allows efforts to be focused on the underlying molecular interaction of sense and antisense RNAs without complications resulting from an altered phenotype. Moreover, a previous study showed that DHFR synthesis was inhibited by approximately 50% in an *in vitro* translation system when antisense RNA was 20-fold that of DHFR mRNA (16), suggesting that antisense DHFR RNA could inhibit DHFR mRNA translation by hybrid arrest.

MATERIALS AND METHODS

Reagents

The Riboprobe Gemini System and RQ1 RNase-free DNase were obtained from Promega (Madison, WI). Lipofectin, horse serum, RPMI-1640, Opti-mem and RNase T1 were purchased from

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GIBCO-BRL (Grand Island, NY). Hybond-N membranes were obtained from Amersham (Arlington Heights, IL). RNase A was obtained from Sigma (St. Louis, Mo). [α - 32 P]dCTP and [α - 32 P]CTP (each 3000 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA). Methotrexate (MTX) was obtained from the Drug Synthesis and Chemistry Branch of the NCI (Rockville, MD). All other reagents used were the highest grade available.

Cell lines and culture conditions

The human KB cell line (ATCC CCL 17), originally derived from an epidermoid carcinoma, was purchased from the American Type Culture Collection. KB-1BT is a KB-derived MTX-resistant cell line which overexpresses DHFR mRNA approximately 250-fold (14). Cells were maintained in the complete medium (RPMI 1640 medium supplemented with 5% heat-inactivated horse serum) at 37°C in a 5% CO₂ atmosphere as previously described (14). Cells were tested for mycoplasma contamination every two months and were consistently found to be negative.

Vectors

Mammalian episomal vectors containing the Epstein-Barr virus (EBV) replication apparatus have been used to express antisense RNA (17, 18). In this study, two EBV-based episomal expression vectors, pCEP4 and pREP4 (both from Invitrogen, San Diego, CA), were employed to express DHFR antisense RNA. Both vectors contain the EBV origin of replication (oriP) and genes coding for an EBV nuclear antigen (EBNA-1) and hygromycin-B resistance. pCEP4 utilizes the cytomegalovirus (CMV) immediate early promoter/enhancer and pREP4 utilizes the Rous sarcoma virus long terminal repeat (RSV-LTR) as the respective promoters to drive transcription of cloned inserts. pGEM-1 was obtained from Promega.

Subcloning antisense DHFR RNA-expressing constructs

To target DHFR mRNA, a variety of DHFR cDNA fragments (Fig 1C) were designed, primarily encompassing portions of the protein coding region (i.e., exons 1 through 6). The 5'UT region was included where exon 1 was present (in the nomenclature of the cloned inserts, exon 1 includes the 5'UT, Fig 1C). Intron I was present in some cDNA fragments as well. Pertinent features of the human DHFR gene and the relative positions of primers used for preparing various DHFR cDNA fragments are illustrated in Fig 1A for reference.

A three-step process was undertaken to generate DHFR cDNA fragments. PCR was carried out under conditions recommended by the supplier of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT). The first two steps are illustrated in Fig 1B. To obtain cDNA fragments containing intron I, the plasmid pLHDIA, which contains DHFR exon 1, intron I and exons 2 through 6 (Will and Dolnick, unpublished results), was used as the template to amplify the initial fragment of DHFR exon 1-intron I-exons 2-6. Subsequently, a cDNA fragment of DHFR 5'UT-exon 1-intron I-exon 2 (ex1-I-ex2, Fig 1B) was added to supply the 5'UT region. The two cDNA fragments were denatured, hybridized and extended under standard PCR conditions without primers to generate the desired DHFR cDNA. To obtain cDNA fragments lacking intron I, pGHDA (14) which contains the complete DHFR coding region (i.e., exon 1 through 6) was used as the template in the first step. In the third step, the cDNA fragments obtained through the first two steps (i.e.,

ex1-I-ex2-6 and ex1-6) were used as templates (Fig 1A, i.e., #352 and #370 for the final fragment ex1-3, #366 and #355 for the final fragment ex2-6) to amplify the desired DHFR cDNA fragments (Fig 1C) under standard PCR conditions. Additionally, ex1-4 was directly amplified from KB-1BT cellular RNA with the primers #352 and #357 using the reverse transcription and PCR protocols previously described (20) (Fig 1C).

These DHFR cDNA fragments were cloned, in the antisense orientation relative to the promoter, at the BamHI and HindIII sites of pREP4 or pCEP4 (21). The structures of the resulting recombinant plasmids are indicated schematically in Fig 1C, with names reflecting the parent plasmid and insert (i.e., pREP4ex1-I, pCEP4ex1-4(1BT), etc.). Some of the cDNA fragments were also cloned into pGEM-1 to allow the preparation of single stranded RNAs by *in vitro* transcription (Fig 1C). The contents of recombinant plasmids were confirmed by restriction analysis.

Transfection

Stable transfection of KB or KB-1BT cells by recombinant plasmids was achieved using the lipofectin-incorporation protocol recommended by the manufacturer, with minor modifications. Lipofectin-plasmid complexes were made by mixing 50 μ g plasmid in 50 μ l H₂O with 50 μ l lipofectin. 5×10^5 cells were incubated with the mixture for 12 hours at 37°C in Opti-mem medium. The medium was then replaced by complete medium and incubation resumed for another 12 hours. At that point, cells were trypsinized and replated at 5-, 15- and 45-fold dilutions. After 24 hours, hygromycin-B was added to a final concentration of 150 μ g/ml. For KB cells, leucovorin (N⁵-formyl tetrahydrofolic acid) was added to 2 μ M to prevent any potential block of cellular metabolism imposed by antisense RNA suppression of DHFR synthesis. Transfection efficiencies were generally in the range of 0.1-1%. Approximately 2 weeks after replating, transfected cells were trypsinized and expanded (uncloned transfectants). In addition, some colonies were picked with cloning rings and expanded (clones). Aliquots of the transfected cells (uncloned transfectants and clones) were frozen. Transfected cell cultures between 5-20 passages were used for further analysis.

Probes and RNA standards

32 P- or 3 H-RNA (used as hybridization probes and quantitation standards, respectively) were synthesized from linearized plasmids (Fig 1D) using the appropriate RNA polymerases and the Riboprobe Gemini kit. The specific activity of 32 P-RNA probes was $2-3 \times 10^8$ dpm/ μ g RNA. 3 H-RNA standards were quantitated by measuring the incorporation of TCA insoluble 3 H-cpm and calculating the amount of 3 H-RNA based on the base composition of the 3 H-transcripts.

DHFR ex1-HaeIII 32 P-DNA was synthesized using a random primer kit (Amersham) and a 146 bp BamHI/HaeIII restriction fragment isolated from pGEM-1ex1-4(1BT) (Fig 1D). The specific activity of the 32 P-probe was $1.5-2 \times 10^9$ dpm/ μ g.

Preparation of cellular RNA

RNA was extracted from log-phase cells using guanidine thiocyanate and purified by ultracentrifugation in CsCl gradients as previously described (22). This procedure does not alter the status of RNA duplexes (see Hybrid duplex RNA assay). Samples were stored at -80°C until use.

Figure 1. Construction of antisense DHFR RNA-expressing vectors. **A)** Organization of the human DHFR gene (19) and locations of primers used in cDNA preparation (not drawn to scale). Open boxes represent exons. Hatched boxes represent 5' or 3' untranslated regions (UT). Solid horizontal lines (some lines are broken) represent introns. The lengths (bp) of segments of the DHFR gene used in preparation of antisense constructs are indicated in italics. Primer sequences are shown along with their numerical designations. Regions of primers with identity to the DHFR gene are underlined and their positions relative to the exons or intron I are indicated with coordinates of intron I or the particular exon involved in parentheses as being either sense (+) or antisense (-) (e.g. position +1 corresponds to the first base of the exon on the sense strand). Restriction sites on primers are indicated as follows: 'E' (EcoRI), 'B' (BamHI), 'N' (NotI), 'SI' (SalI) and 'H' (HindIII). Each primer also contains a 6-base spacer on the 5' end (GATTAAG). **B)** The initial two steps used to generate DHFR templates for antisense insert construction. The first step employed standard PCR conditions using the primers #21 and #355 and the respective plasmids (pLHDIA or pGHDA). Then the cDNA fragment ex1-I-ex2 was added to provide the 5'UT region. The second step of DNA extension proceeded as described in the text. **C)** DHFR cDNA fragments and the recombinant vectors. Schematic diagrams of the DHFR fragments are shown in the left column and the designated names are shown below each fragment. The second column indicates the lengths (bp) of the DHFR cDNA fragments. Open boxes represent exons, hatched boxes represent the 5'UT and horizontal lines indicate intron I. C, R and G represent the vectors pCEP4, pREP4 and pGEM-1, respectively. A '+' indicate a recombinant vector comprising the respective DHFR insert and the parent vector. **D)** ³²P-probes and ³H-RNA standards (see Methods). Lengths are indicated in nt (RNA) or bp (DNA), as are the restriction enzyme sites used to linearize vector templates. Arrows indicate the directions of RNA or DNA products.

Northern blotting

Northern blot analysis was performed essentially as described (21). Briefly, 20 μ g of cellular RNA or known amounts of 3 H-RNA standard were resolved by denaturing gel electrophoresis (1.4% agarose-2.2 M formaldehyde). The correct loading of RNA samples was confirmed after electrophoresis by visual inspection of the ethidium bromide-stained fluorescent samples under ultraviolet light. RNA samples were blotted to Hybond-N membrane overnight in the presence of 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Prehybridization and hybridization were each carried out overnight at 42°C for membranes hybridized with 32 P-DNA probes and at 65°C or 70°C for membranes hybridized with DHFR ex1-HaeIII sense 32 P-RNA or DHFR ex1-4 antisense 32 P-RNA, respectively. After hybridization, membranes were washed twice for 30 minutes each in 2 $\times$ SSC, 0.2% sodium dodecyl sulfate (SDS) at room temperature; then twice for 30 minutes each in 0.1 $\times$ SSC, 0.2% SDS at 55°C for membranes hybridized with DNA probes and at 70°C or 75°C for membranes hybridized with DHFR ex1-HaeIII sense 32 P-RNA or DHFR ex1-4 antisense 32 P-RNA, respectively.

Hybrid duplex RNA assay

The hybrid duplex RNA assay was adopted from previously described protocols (5, 21). To eliminate possible DNA contamination in isolated cellular RNA, RNA samples (30 μ g each) were subjected to DNase digestion in a 30 μ l reaction containing 2 units RQ1 DNase, 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂ and 10 mM NaCl at 30°C for 30 minutes. To the DNase treated samples was added 300 μ l of a solution containing 0.3 M NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 12 μ g RNase A and 8 units RNase T1. The mixture was incubated at 30°C for 1 hour. 8.5 μ l 20% SDS and 5 μ l 20 μ g/ μ l proteinase K were then added and the mixtures were incubated for 30 minutes at 37°C. 30 μ g of *Escherichia coli* tRNA was then added to each tube. Samples were phenol-chloroform extracted and RNA was ethanol precipitated. The precipitated RNA was dissolved in 30 μ l hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES pH 6.4) containing 2 $\times$ 10⁵ cpm DHFR sense 32 P-RNA, heated at 85°C for 10 minutes, then immediately transferred to a 50°C bath and incubated for 14–18 hours. Samples were then subjected to a second ribonuclease treatment under the conditions described as above. Known amounts of DHFR ex1-I-ex2-6 or ex1-4 antisense 3 H-RNAs, each with 30 μ g *E. coli* tRNA, were subjected to the same treatment in parallel, except that buffer was substituted for the ribonucleases in the first ribonuclease-treatment step. RNAs were then dissolved in 5 μ l gel loading buffer (80% formamide, 10 mM Tris-HCl pH 7.5, 0.1% bromophenol blue), heated at 85°C for 5 minutes and resolved by denaturing gel electrophoresis (3.5% polyacrylamide-7 M urea). Gels were dried, autoradiograms obtained using Kodak XAR X-ray film, and quantitation was performed as described (see Quantitation).

A control reconstitution experiment was conducted to evaluate whether the RNA preparation procedure or the hybrid duplex RNA assay affected the measurement of hybrid duplex RNA. 200 fmoles each of DHFR ex1-6 sense 3 H-RNA and DHFR ex1-I-ex2-6 antisense 3 H-RNA were combined in 60 μ l hybridization buffer and heated at 85°C for 10 minutes. The solution was divided into two 30 μ l aliquots. One aliquot was immediately placed on ice ('single-stranded'). The other aliquot

was incubated at 50°C overnight and then placed on ice ('hybridized'). 25 μ l of either single-stranded or hybridized 3 H-RNA was added to KB-1BT cells and RNA was extracted as described. Aliquots were then subjected to the hybrid duplex RNA assay (see Results and Fig 3).

Quantitation of RNA species

DHFR mRNA and antisense RNA levels obtained from Northern blots were quantitated using an ImageQuant densitometer (Molecular Dynamics). Autoradiograms were obtained using Kodak X-AR X-ray film with multiple exposures to ensure signal intensity was in the linear range. DHFR mRNA levels were determined by comparing their signal intensities with those of known amounts of a DHFR ex1-6 sense 3 H-RNA standard included on the gels, after probing of filters with DHFR ex1-4 antisense 32 P-RNA. Similarly, DHFR antisense RNA levels were measured by comparing their signal intensities with those of known amounts of a DHFR ex1-I-ex2-6 or ex1-4 antisense 3 H-RNA standard, after probing with DHFR ex1-HaeIII sense 32 P-RNA. Results are expressed as amoles DHFR mRNA or antisense RNA per μ g cellular RNA.

Quantitation of sense:antisense hybrid duplex RNAs from the hybrid duplex RNA assay was accomplished by scanning dried polyacrylamide gels with an ImageQuant phosphorimager (Molecular Dynamics). The sums of all duplex species detected in each lane were compared with the signals of known amounts of DHFR ex1-I-ex2-6 or ex1-4 antisense 3 H-RNA standards separately. Results are expressed as amoles full-length hybrid duplex RNA (i.e., exon 1 for pCEP4*ex1-I* transfectants and exons 1-4 for pCEP4*ex1-4(1BT)* transfectants) per μ g cellular RNA.

The levels of intracellular DHFR mRNA, antisense RNA and hybrid duplex RNA can be described by the following equations:

$$[S] = [S_{ss}] + [S:AS] \quad (1)$$

$$[AS] = [AS_{ss}] + [S:AS] \quad (2)$$

In the equations [S], [AS] and [AS:S] represent levels of DHFR mRNA, antisense RNA and hybrid duplex RNA, respectively, and the subscript 'ss' denotes single-strandedness.

A hybrid index ($H_{s:as}$) was devised as defined by the equation:

$$H_{s:as} = \frac{[S:AS]}{[S_{ss}][AS_{ss}]} \quad (3)$$

In this equation RNAs are expressed as mol/ μ g cellular RNA. [S:AS] is determined from the hybrid duplex RNA assay; [S_{ss}] and [AS_{ss}] are obtained from equations 1 and 2 by subtracting [S:AS] from [S] and [S:AS] from [AS], respectively. Since [S:AS] was found to be very low compared to [S] (i.e., less than 0.14%) in transfected cells, [S_{ss}] is essentially equal to [S].

RESULTS

Expression of DHFR antisense RNAs in transfectants

The expression efficiency of the recombinant vectors in transfected cells was evaluated (Fig 2A). Cells transfected with recombinant pCEP4 vectors expressed the expected DHFR antisense RNA species (ex1-I-ex2-6, lane 1 and ex1-I, lane 3). However, in cells transfected with recombinant pREP4 vectors, antisense RNA was either non-detectable (ex1-I-ex2-6, lane 2) or migrated with an anomalously high molecular weight (ex1-I, lane 4). This poor and anomalous expression with the pREP4

Figure 2. Steady-state antisense RNA levels. **A)** Comparison of DHFR antisense RNA expression in transfected cells. RNA (20 μ g each) from uncloned KB transfectants carrying pCEP4 $ex1-I-ex2-6$ (lane 1), pREP4 $ex1-I-ex2-6$ (lane 2), pCEP4 $ex1-I$ (lane 3) or pREP4 $ex1-I$ (lane 4) were resolved by denaturing gel electrophoresis. Northern blotting was performed as described. The membrane was hybridized with DHFR exon 1-HaeIII ³²P-DNA. The vectors (pCEP4 or pREP4) used for antisense construction are indicated above the panel and the positions of the major species of DHFR mRNA (s) in KB cells and antisense RNAs (as) are indicated to the left of the panel. Molecular weights of the RNA species are indicated in kb on the right. **B)** Comparison of DHFR antisense RNA levels from transfectants containing different recombinant pCEP4 vectors. RNA (20 μ g each) from uncloned KB transfectants carrying pCEP4 $ex1-I$ (lane 5), pCEP4 $ex1-3$ (lane 6), pCEP4 $ex1-4$ (lane 7), pCEP4 $ex1-5$ (lane 8) and pCEP4 $ex1-6$ (lane 9) were resolved by denaturing gel electrophoresis and blotted as described. The membrane was hybridized with DHFR exon 1-HaeIII sense ³²P-RNA. Molecular weights for 28S rRNA (4.7 kb), DHFR $ex1-3$ (0.5 kb) and $ex1-I$ (0.8 kb) antisense RNAs are indicated to the left of the panel.

Figure 3. Fidelity of the hybrid duplex RNA assay. A test of the fidelity of the hybrid duplex RNA assay was conducted as described (see Methods). 'hybridized' represent the use of hybridized single-stranded DHFR $ex1-6$ sense ³H-RNA and DHFR $ex1-I-ex2-6$ antisense ³H-RNA. 'single-stranded' represent the use of denatured single-stranded DHFR $ex1-6$ sense ³H-RNA and DHFR $ex1-I-ex2-6$ antisense ³H-RNA. 'RNA extraction' indicates whether the control RNA mixtures ('hybridized' or 'single-stranded') were added to KB-1BT cells for standard RNA preparation. '1st digest' and '2nd digest' represent the first and second ribonuclease digestions. In the second digestion, DHFR $ex1-HaeIII$ sense ³²P-RNA was used as the probe and the expected protected fragment is indicated to the left.

vectors can likely be ascribed to the combination of a low level of transcription and transcriptional read-through. The data show that pCEP4 is superior to pREP4 in generating the expected antisense RNAs. KB-1BT cells transfected with the same recombinant pCEP4 vectors showed essentially the same

Figure 4. DHFR antisense RNA and mRNA levels in KB-1BT transfectants. **A)** DHFR antisense RNA levels. RNAs (20 μ g each) from different transfected cell populations were resolved by denaturing gel electrophoresis and transferred to Hybond-N membranes as described. The membranes were hybridized with DHFR exon 1-HaeIII sense ³²P-RNA (left panel) or DHFR exons 1-4 sense ³²P-RNA (right panel). The lanes in the left panel are for cells transfected with pCEP4 $ex1-I$: #4 (lane 1), #5 (lane 2), #17 (lane 3), #9 (lane 4), #9A (lane 5), #9B (lane 6) and #9C (lane 7). The lanes in the right panel are for cells transfected with pCEP4 $ex1-4$: #10A (lane 8), #10C (lane 9), #10D (lane 10), #10E (lane 11), #10F (lane 12), #10G (lane 13), #11A (lane 14), #11D (lane 15), #11E (lane 16), #11I (lane 17) and #10 (lane 18). The transfected cells are specified above the panels (i.e., 'cells') as either uncloned transfectants (i.e., 'uncloned') or clones (i.e., 'cloned'). In the panels, positions of the major species of DHFR antisense RNAs (as $ex1-I$ in left panel and as $ex1-4$ in right panel) and 18S and 28S rRNAs are indicated. **B)** DHFR mRNA levels in KB-1BT transfectants used in hybrid duplex RNA determination. RNAs (0.125 μ g each) from KB-1BT and KB-1BT transfectants were resolved by denaturing gel electrophoresis and transferred to Hybond-N membranes. The membranes were hybridized with DHFR exons 1-4 antisense ³²P-RNA. The lanes correspond to KB-1BT (lane 1), #5 (lane 2), #9 (lane 3), #17 (lane 4), #10 (lane 5), #4 (lane 6), #9A (lane 7), #9C (lane 8), #10C (lane 9), #10D (lane 10), #10E (lane 11), #10G (lane 12) and #11D (lane 13). The sizes (in kb) of the three species of DHFR mRNA in KB-1BT cells are indicated to the right.

expression pattern (data not shown). Therefore, recombinant pCEP4 vectors were selected for further studies. Human cells typically contain 3.6 and 1.0 kb DHFR mRNA species differing in the lengths of the 3' non-coding region (15). The level of 3.6 kb DHFR mRNA was fairly constant in the different KB transfectants (Fig 2A, lanes 1-4). The 1.0 kb DHFR mRNA species is not visible in the blot shown in Fig 2A as it is present at much lower levels. The amount of DHFR mRNA per cell was determined from the Northern blot as described. Based upon a cellular content of 1.5×10^{-5} μ g/cell for KB cells (23), there are estimated 200 copies of DHFR mRNA per cell, consistent with the value obtained by quantitative PCR (20). DHFR $ex1-I$ antisense RNA level (Fig 2A, lane 3) is estimated to be approximately 15-fold that of DHFR mRNA.

Fig 2B illustrates that the steady-state levels of DHFR antisense RNAs containing different antisense exon sequences varied widely. Using DHFR antisense $ex1-5$ RNA level (lane 8) as

Figure 5. Hybrid duplex RNA levels in KB-1BT transfectants. **A)** Hybrid duplex RNA levels in pCEP4ex1-I transfectants. ^3H -RNA standard and cellular RNA samples were subjected to hybrid duplex RNA assay as described and the final ethanol precipitated RNAs were resolved by denaturing polyacrylamide gel electrophoresis and visualized on autoradiogram. Lanes correspond to: 0.1 fmole DHFR ex1-I-ex2-6 antisense ^3H -RNA standard (lane 1, with no first ribonuclease digestion); cellular RNAs from the transfectants, #4 (lane 2), #5 (lane 3), #17 (lane 4), #9 (lane 5), #9A (lane 6) and #9C (lane 7); probe controls, 2×10^5 cpm DHFR exon1-HaeIII sense ^{32}P -RNA in each of lanes 8 and 9, in lane 9 the sample underwent a mock ribonuclease digestion and only 2% of the original sample was loaded to the gel; ^{32}P -end labeled MspI-digested pBR322 DNA size markers (lane 10). The positions of the hybrid duplex RNA (d) and undigested probe (p) are indicated to the left and the sizes of selected molecular weight markers (nt) are indicated to the right. **B)** Hybrid duplex RNA levels in pCEP4ex1-4 transfectants. ^3H -RNA standard and cellular RNAs were subjected to the hybrid duplex RNA assay as described and the final ethanol precipitated RNAs were resolved by denaturing polyacrylamide gel electrophoresis and visualized on an autoradiogram. Lanes correspond to: 0.16 fmole antisense ^3H -RNA standard (lane 1, with no first ribonuclease digestion); cellular RNAs from transfectants, #10 (lane 2), #10C (lane 3), #10D (lane 4), #10E (lane 5), #10G (lane 6) and #11D (lane 7); probe controls, 2×10^5 cpm DHFR ex1-4 sense ^{32}P -RNA in each of lanes 8 and 9, in lane 9 the sample underwent a mock ribonuclease digestion and only 2% of the original sample was loaded to the gel; lane 10 contains the same sample as that in lane 10 of panel A. The positions of selected molecular weight markers (nt) are indicated to the right.

a standard, the relative levels of antisense RNAs from constructs containing exon 5 or exons 5 and 6 ranged from 1 to 1.4-fold (lanes 8–9), while those from constructs lacking exon 5 or exons 5 and 6 ranged from 4.0 to 7.6-fold (lanes 5–7). The trend for lower antisense RNA levels from constructs containing exons 5 or 6 is also true for ex1-I-ex2-6 (lane 1 vs. lane 3 in Fig 2A), ex2-5 and ex2-6 (data not shown). Southern blot analysis of DNA from transfected cells indicated that despite variations in antisense RNA levels, vector copy numbers are approximately 1 copy/cell (data not shown), ruling out vector copy number as a possible cause for such variations. In Fig 2B, minor antisense RNA species with higher molecular weight (i.e., approximately 4.5–4.7 kb) are also evident. Based on the antisense RNA levels found, the recombinant vectors pCEP4ex1-I and pCEP4ex1-4(1BT) were chosen for further study.

Fidelity of the hybrid duplex RNA assay

The utility of a hybrid duplex formation assay depends on how it reflects the status of hybrid duplex RNA *in situ*. To address this issue, KB-1BT cells were combined with either single or double-stranded ^3H -RNAs and the amount of duplexed ^3H -RNAs was measured using the hybrid duplex assay (see Methods). Results are shown in Fig 3. The 146 nt DHFR ex1-HaeIII sense ^{32}P -RNA, detected after nuclease treatment, is the size expected for the duplex RNA species comprising DHFR ex1-6 sense and ex1-I-ex2-6 antisense ^3H -RNAs. Samples

Figure 6. Hybrid duplex RNA levels as a function of single-stranded antisense RNA levels. The levels of hybrid duplex RNA of selected KB-1BT transfectants are plotted as a function of the corresponding single-stranded (ss) DHFR antisense RNA. Closed triangles, pCEP4ex1-4 transfectants; Open circles, pCEP4ex1-I transfectants.

which were not subjected to the first RNase digestion (i.e., lanes 1, 3, 5 and 7) represent the total amount of DHFR ex1-I-ex2-6 antisense RNA present while lanes 2, 4, 6 and 8 correspond to the amount of DHFR ex1-I-ex2-6 antisense ^3H -RNA present in hybrid duplex form. A comparison of lanes 1 and 2 indicates that approximately 50% of the hybridized ^3H -RNA added to the cells was present in duplex form (the amount of 146 nt band in lane 2 is 49% that in lane 1). The RNA extraction step did not significantly alter the estimated percent hybrid duplex (the amount of 146 nt band in lane 4 is 53% of that in lane 3). Conversely, there was no hybrid duplex detected from cells to which denatured single-stranded ^3H -RNA was added (lane 8). The weak signal in lane 6 is the result of sample contamination from adjacent lanes. Together, these results indicate that the hybrid duplex RNA assay likely reflects the *in situ* duplex status of cellular RNA.

Sense, antisense and hybrid duplex RNA levels in transfected cells

Preliminary assays with transfectants expressing DHFR ex1-I antisense RNA indicated that hybrid duplex RNA was not detectable in KB transfectants but was detectable in KB-1BT transfectants (data not shown). This suggested that hybrid duplex RNA formation was probably a function of both the sense and antisense RNA levels and that steady state hybrid duplex RNA levels might be described by a mass action type equation.

DHFR antisense RNA levels in KB-1BT cells transfected with the pCEP4ex1-I or pCEP4ex1-4(1BT) were evaluated by Northern blot analysis (Fig 4A). The major species of antisense RNAs had the expected sizes for the predicted processed transcripts (i.e., ex1-I or ex1-4) plus a poly A tail. Many samples (e.g., lanes 4, 7, 8, 9, 10, 11, 15, 18, etc.) showed additional minor species of higher molecular weight which probably reflect the presence of read-through transcripts. The wide spectrum of antisense RNA levels was exploited to determine how the levels of antisense and sense RNAs were related to the levels of hybrid duplex RNAs.

DHFR mRNA levels in the KB-1BT transfectants chosen for study (#4, 5, 17, 9, 9A and 9C for pCEP4ex1-I transfectants;

Table 1. Levels of DHFR sense, antisense and hybrid duplex RNAs; values of $H_{s:as}$

RNA ^a	Duplex [S:AS] ^b	Antisense RNA [AS] ^c	[AS] _{ss} ^d	% ^e	DHFR RNA [S] ^f	$H_{s:as}$ ^g
ex1-I						
#4	0.9	14.0	13.1	6.4	5.1×10^3	1.3
#5	1.8	27.0	25.2	6.7	4.4×10^3	1.6
#17	1.3	15.0	13.7	8.3	5.5×10^3	1.7
#9	3.3	38.0	34.7	8.6	5.4×10^3	1.8
#9A	0	0	0	0	5.4×10^3	—
#9C	9.5	177.5	168.0	5.3	5.6×10^3	1.0
average ^h						1.5 ± 0.3
ex1-4						
#10	7.9	31.0	23.1	25.3	5.5×10^3	6.2
#10C	6.5	25.5	19.0	24.3	5.3×10^3	6.5
#10D	2.4	6.5	4.1	36.9	5.4×10^3	10.8
#10E	1.7	8.0	6.3	20.6	5.3×10^3	5.1
#10G	0.7	0	0	0	4.1×10^3	—
#11D	5.8	29.5	23.7	19.5	5.3×10^3	4.6
average ^h						6.6 ± 2.5

^aThe transfectant RNAs analyzed.^bThe average levels of hybrid duplex RNA (amol/ μ g, full-length equivalent) determined from two separate hybrid duplex RNA assays.^cThe average levels of DHFR antisense RNA (amol/ μ g) determined from two separate Northern blots.^dThe levels of single-stranded antisense DHFR RNA (amol/ μ g) determined by subtracting [S:AS] from [AS].^eThe percent of full-length equivalent DHFR antisense RNA present as hybrid duplex.^fThe average levels of DHFR mRNA (amol/ μ g) determined from two separate Northern blots.^gThe hybrid duplex index ($\times 10^3$ μ g/mol).^hThe average $H_{s:as} \pm$ standard deviation ($\times 10^{13}$ μ g/mol).

and #10, 10C, 10D, 10E, 10G and 11D for pCEP4ex1-4(1BT) transfectants) were determined by Northern blot analysis (Fig 4B). The observation of three DHFR mRNAs species (1.4, 1.0, 0.8 kb) in KB-1BT cells is consistent with previous observations (14). DHFR mRNA levels are relatively constant for these samples. Values of DHFR antisense RNA and mRNA levels are presented in Table 1.

A typical hybrid duplex RNA analysis for a number of transfectants is shown in Fig 5. For the pCEP4ex1-I transfectants (Fig 5A, lanes 2-7), the major protected RNA species are a set of three bands clustered in the 130-146 nt size range, equal or close to the length of the DHFR sequence contained in the probe (146 nt ex1-HaeIII, see Fig 1D), suggesting hybrid duplex RNA formation between the exon 1 region of DHFR mRNA and ex1-I antisense RNA. The multiple banding pattern is likely due to the presence of alternate transcription initiation sites in the DHFR gene as previously reported for another DHFR gene-amplified KB cell line (13). In transfectant #9C (lane 7), some species with lower molecular weight were also detectable, which may represent partial duplex formation (see Discussion). The undigested ³²P-probe migrated as an expected 170 nt fragment (lane 9). The size difference between the full-length probe (178 nt, lane 9) and the 146 nt protected fragment results from digestion of the transcribed polycloning site which can not hybridize to DHFR mRNA. The residual undigested ³²P-probe (lane 8) does not affect quantitation of hybrid duplex RNA since it is well separated from the duplex bands.

Detection of hybrid duplex RNA in the pCEP4ex1-4(1BT) transfectants is illustrated in Fig 5B. In addition to the fully protected 440 nt species (DHFR ex1-4), other protected RNA bands are also observed (lanes 2-7), clustering in the 260-280 nt, 140-150 nt and 110-120 nt size ranges (see Discussion). The difference between the length of the protected 440 nt DHFR

ex1-4 sequence (prominent in lanes 2, 3, 7) and the 472 nt full-length probe (lane 9) derives from digestion of the 32 nt plasmid polycloning site present in the probe. Undigested probe migrated as expected (472 nt, Fig 5B lane 9) and ribonuclease digestion of the probe was complete (lane 8). Results of the hybrid duplex assay are presented in Table 1.

The data in Table 1 indicate that although antisense RNA levels vary widely, the percentage of antisense RNA present in hybrid duplexes is in a narrow range, comprising approximately 5-9% of the DHFR ex1-I antisense RNA or 20-37% of the DHFR ex1-4 antisense RNA in transfectant cellular RNA. The hybrid duplex RNA level as a function of the antisense RNA level for ex1-I and ex1-4 is presented in Fig 6. As illustrated for transfectants expressing either ex1-I or ex1-4 antisense RNA, hybrid duplex RNA levels are a linear function of the single-stranded DHFR antisense RNA levels. A hybrid duplex index, $H_{s:as}$, was calculated using equation 3 to describe the interrelationship of DHFR mRNA, antisense RNA and hybrid duplex RNA. Calculated $H_{s:as}$ values are presented in Table 1.

DISCUSSION

Antisense RNA-mediated phenotypic effects in cells are believed to derive from the ability of antisense RNAs to form hybrid duplexes with mRNA. Therefore, characterization of hybrid duplex RNAs is of fundamental importance to the understanding of the mechanism of antisense RNA effects. Although a few studies have reported the detection of hybrid duplexes in mammalian cells (5, 6, 8) and have suggested that a large excess of antisense RNA may be needed to obtain phenotypic effects, little information exists concerning the quantitative interrelationship of sense, antisense and hybrid duplex RNAs. This study was undertaken to explore that quantitative interrelationship.

Initial attempts to develop efficient expression vectors demonstrated pCEP4 to be superior to pREP4 with respect to the overall quality and steady-state levels of antisense RNA (Fig 2A). The data (Fig 2B) also suggest an element(s) in exon 5 and/or exon 6 adversely affects the steady state levels of the processed RNA transcripts containing these regions. The variations in antisense RNA levels between constructs containing exon 5 or 6 and those without these sequences are not due to differences in insert lengths (e.g. 520 bp ex1-I and 556 bp ex1-5 in Fig 2B, lane 5 and lane 8, respectively). This expression pattern strongly suggests that when transcribed in the antisense orientation, DHFR exon 5 (and possibly exon 6) alters transcription, post transcriptional processing, or RNA stability, leading to lower steady-state levels of the corresponding antisense RNAs. The presence of antisense exon 5 or 6 in antisense DHFR constructs may explain the failure to obtain significant levels of DHFR antisense RNA in an earlier study (5).

Based on these observations, pCEP4ex1-I and pCEP4ex1-4(1BT) were chosen for subsequent studies. KB cells transfected with pCEP4ex1-I accumulated antisense RNA levels up to 15-fold that of DHFR mRNA. However, sense and antisense RNA interactions were found to be either absent or minimal as determined either phenotypically (i.e., no change in MTX cytotoxicity or DHFR enzymatic activity when transfectants were compared with mock transfectants) or by the hybrid duplex RNA assay (data not shown).

Because KB-1BT cells have DHFR mRNA levels about 250-fold that of the parental KB cells, it was hypothesized that hybrid duplexes would more likely be detectable in KB-1BT cells. Based on the antisense RNA levels determined by Northern analysis, twelve different KB-1BT transfectants, six containing pCEP4ex1-I and six containing pCEP4ex1-4(1BT), were selected and analyzed for the steady-state levels of DHFR mRNA, antisense RNA and hybrid duplex RNA.

Most of the hybrid duplex formed between DHFR mRNA and ex1-I antisense RNA was of a size representative of the ex1-HaeIII region (i.e., exon 1, see Fig 5A). However, the hybrid duplexes formed between DHFR mRNA and ex1-4 antisense RNA exhibited a more complex pattern (Fig 5B). The exact reasons for this complex pattern are not clear, but it does not appear to be an artifact resulting from the hybrid duplex RNA assay since the DHFR antisense ex1-4 ³H-RNA standard was fully protected from the second ribonuclease digestion in the assay (Fig 5B, lane 1). The complex pattern is not likely an outcome of preexisting sequence mismatches between DHFR mRNA and the vector-expressed ex1-4 antisense RNA in KB-1BT cells since control hybridization of DHFR ex1-4 antisense ³²P-RNA with KB-1BT RNA yielded a fully protected 440 nt species after RNase digestion (data not shown). It is conceivable that the complex banding pattern results from steric hindrance associated with RNA secondary structures *in situ* or from proteins interacting with the RNA regions involved in hybrid duplex formation (i.e., exons 1-4) such that DHFR mRNA and ex1-4 antisense RNA are not fully accessible to each other. It is also possible that the complex banding pattern results from modifications undergone by hybrid duplex RNAs *in situ* which affect local duplex stability. For example, RNA duplex unwindase is an apparently ubiquitous enzyme that deaminates adenosine residues in RNA duplexes, thus destabilizing and unwinding RNA duplexes (24-27). The action of this enzyme could conceivably be responsible for the complex banding pattern observed in the hybrid duplex RNA assay without an obvious alteration in Northern blot results.

Another possibility is that multiple antisense RNAs could hybridize with a single mRNA molecule or vice versa. This would lead to the formation of discontinuous duplex molecules susceptible to nuclease degradation in the hybrid duplex RNA assay. It is calculated that 5-9% of DHFR ex1-I or 20-37% of DHFR ex1-4 antisense RNAs were present as hybrid duplexes in transfected cells. Due to the nature of the quantitation method, it is not clear whether the hybrid duplex RNA species of 260-280 nt, 140-150 nt and 110-120 nt in pCEP4ex1-4(1BT) transfectants (Fig 5B) arose from different antisense RNA molecules or from different regions of the same antisense RNA molecules. The direct implication is that the percentage of antisense RNA molecules which participated in hybrid duplex formation may be equal to (if duplex bands were from the same antisense RNA molecules) or greater than (if duplex bands were from different antisense RNA molecules) the calculated percentage.

In pCEP4ex1-I or pCEP4ex1-4(1BT) transfectants, hybrid duplex RNA levels are a linear function of single-stranded antisense RNA levels. Introduction of the hybrid duplex index ($H_{s:as}$) represents an approach towards the characterization of hybrid duplex formed between sense and antisense RNAs *in situ*. The narrow range of $H_{s:as}$ in different transfectants expressing either DHFR antisense RNA (i.e., $1.0-1.8 \times 10^{13}$ μ g/mol for ex1-I and $4.6-10.8 \times 10^{13}$ μ g/mol for ex1-4) and the difference in the values of $H_{s:as}$ between transfectants expressing DHFR ex1-I and ex1-4 antisense RNAs suggest that the value of $H_{s:as}$ is characteristic of a particular sense:antisense RNA interaction.

While the calculated $H_{s:as}$ values are not easily comparable to data from other antisense RNA studies, the lack of hybrid duplex RNA detection in KB transfectants and the fractional amounts of antisense RNA present in hybrid duplex form in KB-1BT transfectants imply that hybrid duplex RNA formation between DHFR mRNA and antisense RNA may be inefficient. Solution hybridization in a complex nucleic acid population follows slow second order kinetics (28-30). In a number of respects RNA:RNA hybrid duplex *in situ* reflects the behavior of nucleic acid hybrid formation in solution. The observed low hybrid duplex RNA level and corresponding low $H_{s:as}$ values in this study may be a reflection of intracellular hybridization kinetics in general. The higher percentage of antisense molecules in duplex and greater $H_{s:as}$ values for DHFR ex1-4 antisense RNA as opposed to those for ex1-I antisense RNA can be interpreted as the ex1-4 antisense RNA having a higher hybridization rate constant (k). This result is compatible with k being proportional to the square root of the complementary sequence length in solution hybridization kinetics (30). However, it is likely that the process of intracellular hybrid duplex RNA formation is affected by various cellular factors. Although the levels of single-stranded DHFR mRNA and antisense RNA, $[S_{ss}]$ and $[AS_{ss}]$, were used in equation 3 to calculate $H_{s:as}$, it is possible that only a fraction of single-stranded sense and antisense RNAs is free to participate in hybrid duplex formation. In consideration of all these factors, it is not surprising that overall intracellular hybrid duplex formation appears to be an inefficient process.

An interesting extrapolation can be made from the $H_{s:as}$ values obtained, with regard to the potential for the formation of DHFR hybrid duplex RNA in KB transfectants. The average $H_{s:as}$ values are 1.5×10^{13} μ g/mol (ex1-I) and 6.6×10^{13} μ g/mol (ex1-4). Assuming these $H_{s:as}$ values remain invariant over a wide range of DHFR mRNA and antisense RNA levels and the

DHFR mRNA level in KB transfectants is 24 amol/ μ g RNA, in order to drive 50% of the DHFR mRNA in KB cells into hybrid duplex, 6.7×10^4 amol/ μ g (2778-fold the level of DHFR mRNA) ex1-I antisense RNA or 1.5×10^4 amol/ μ g (631-fold the level of DHFR mRNA) ex1-4 antisense RNA would be required. These calculated ratios of antisense RNA to sense RNA are similar to those found in other antisense RNA studies (5, 33-36). This study suggests that in cells, interactions of sense and antisense RNAs may be inefficient in general and the efficiency may vary depending on the length and/or primary structure of the RNAs involved.

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