Production of antisense RNA by transfected expression vectors has been used to down-regulate specific genes (8). Although nuclear run-on transcription of initiated RNA transcripts in isolated nuclei can be used to measure transcription of endogenous genes, use of double-stranded cDNA probes to detect radiolabeled transcripts does not allow distinction between specific mRNAs and their complementary antisense RNA sequences. Although the ability to discriminate between sense and antisense transcription has been reported using single-stranded M13 DNA (3,4), this method requires lengthy cloning, restriction analysis and sequencing in a specialized vector, followed by laborious isolation of single-stranded template DNA. We describe here a modification of existing nuclear run-on transcription measurement methods in isolated nuclei that allows concurrent measurement of sense and antisense RNA transcription in the same nuclei, using synthetic oligonucleotides as probes. We measured, in a human breast cancer cell line (MCF-7), transcription of thymidylate synthase (TS), glyceraldehyde phosphate dehydrogenase (GAPDH), 18S rRNA genes and transiently transfected vectors (pAS/6-9 and pAS/191-1) designed to produce antisense molecules complementary to TS mRNA sequences. Although the RNAs synthesized were approximately 350 bases in length, only 30 bases of each were antisense to specific regions of the TS mRNA. pAS/6-9 and pAS/191-1 targeted TS mRNA close to the translation start site (nucleotides +6 to +35) and the exon 1/exon 2 boundary (nucleotides +191 to +220), respectively (Figure 1 and Figure 2). Antisense-expressing double-stranded oligonucleotides were cloned into the HindIII/XbaI site of the eukaryotic expression vector pRC/cytomegalovirus (CMV) and transfected into $1.5 \times 10^6$ MCF-7 cells using LipoFECTAMINE™ (Life Technologies, Burlington, ON, Canada). Control cells were transfected with the pRC/CMV vector without insert. Relative transcription rates were determined by nuclear run-on transcription in isolated nuclei as described previously (1,5). MCF-7 nuclei were isolated 48 h after transfection and stored in liquid nitrogen until use. Then $2 \times 10^7$ thawed nuclei were incubated in 180 µL of reaction mixture for 30 min at 30°C in the
presence of [α-32P]UTP. 32P-labeled RNA was isolated using RNeasy® silica gel-based membrane separation columns (Qiagen, Chatsworth, CA, USA). For hybridizations, which were performed in a total volume of 2 mL, 2 × 10^6 cpm were used. To distinguish between TS mRNA and antisense RNA molecules, target DNA (immobilized in triplicate dots, 2 µg per dot, on nylon filters [Hybond™; Amersham, Oakville, ON, Canada] without baking or ultraviolet irradiation) consisted of single-stranded synthetic oligonucleotides rather than TS cDNA.

Single-stranded TS oligonucleotide probes are numbered according to the sequence available in GenBank® Accession No. X02308. Bases in boldface form part of restriction endonuclease sites and are not sense or antisense TS sequences as follows: TS cDNA nucleotides +6 to +35 (near the translation start site) sense (JK-5): CTAGAGCTACAGCCTGAGAGATGAATCCC-TCTGCA and antisense (JK-4): AGCCTTGAGGGGATTCATCTCTCA GGCTGTAGCT.

Unlabeled complementary DNA probes for GAPDH mRNA and 18S rRNA were immobilized on filters to capture radiolabeled GAPDH and 18S rRNA transcripts. Data presented in Figure 1 (phosphor image) and Figure 2 (right panel) indicate that GAPDH mRNA and 18S rRNA have been transcribed at roughly the same rate irrespective of the presence or absence of antisense TS RNA expression vector. Figure 1 and Figure 2 (left panel) show that antisense TS expression vectors producing antisense RNA that have bound to probes JK-5 or JK-3 are transcribed at a high rate in transfected cells. However, cells expressing antisense TS RNA from vector pAS/6-9 (complementary to the TS mRNA translation start site) had elevated transcription from constitutive TS genes (detected by increased binding to driver DNAs JK-2 and JK-4). Cells harboring and transcribing vector pAS/191-1, on

![Figure 1. Phosphor-imager visualization of nuclear run-on transcription of constitutive genes and transfected expression vectors in cultured human MCF-7 breast cancer cells.](image)

Specific radiolabeled RNAs were transcribed, in isolated cell nuclei, over a 30-min period from constitutive genes (TS mRNA, GAPDH mRNA, 18S rRNA) or from transiently-transfected foreign DNA vectors (antisense thymidylate synthase [AS TS] RNA). “DNA probe” indicates the unlabeled driver DNA immobilized, in three identical dots, on filters: the probe binds radiolabeled RNA produced during the 30-min transcription period. JK-2 and JK-4 are different probes that specifically bind thymidylate synthase mRNA, but not antisense TS RNA. JK-5 and JK-3 are different probes that specifically bind to antisense RNA to TS, but not to TS mRNA. cDNA probes for “housekeeping” genes (GAPDH, 18S rRNA) are capable of binding to both antisense RNA and mRNA: in this case, only mRNAs are detected. “RNA synthesized” indicates the RNA whose transcription is being detected by each DNA probe. “Transfected DNA” (pAS/6-9; for example) refers to transfected vectors from which foreign RNA is transcribed. “pRC/CMV” refers to cells transfected with parent vector alone without antisense RNA-expressing sequences. “pAS/6-9” and “pAS/191-1” refer to cells transfected with two different pRC/CMV-based vectors transcribing RNAs antisense to different regions of human TS mRNA (near the translation start site and the exon 1/exon 2 boundary) respectively. Radiolabeled RNA (2 × 10^6 cpm) synthesized from cells harboring each of these vectors was hybridized in three separate bags (2 mL of hybridization solution per bag), as described in the text. The darkness of the phosphor image indicates the amount of radiolabel and is directly proportional to the level of transcription of each constitutive gene or transfected foreign DNA.
the other hand, did not transcribe constitutive TS genes at a higher rate (indicated by low-level binding to JK-2 and JK-4). Therefore, antisense RNA directed at the thymidylate synthase mRNA translation start site, but not other region of TS mRNA, stimulated transcription of constitutive TS genes.

Using this method, we were successful in distinguishing constitutive mRNA from antisense RNA transcribed from transfected vectors (Figure 1 and Figure 2). Short, easily synthesized and purified single-stranded 30-mer oligonucleotides rather than longer (500-mer and longer) single-stranded DNAs, derived from M13, worked well as probes for radiolabeled RNAs. Given the increasing use of antisense RNA expression vectors to down-regulate specific genes in eukaryotic cells and recent reports of naturally occurring antisense RNA in eukaryotic cells (2,3,6,7), this method will be useful in assessing the accumulation of antisense RNA within cells after transfection of appropriate expression vectors, the effect of that accumulation on transcription of constitutive genes to produce complementary mRNA, and the presence or absence of naturally occurring antisense RNA sequences.

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