Two Distinct Promoters Drive Transcription of the Human D1A Dopamine Receptor Gene*

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The human D1A dopamine receptor gene has a GC-rich, TATA-less promoter located upstream of a small, noncoding exon 1, which is separated from the coding exon 2 by a 116-base pair (bp)-long intron. Serial 3'-deletions of the 5'-noncoding region of this gene, including the intron and 5'-end of exon 2, resulted in 80 and 40% decrease in transcriptional activity of the upstream promoter in two D1A-expressing neuroblastoma cell lines, SK-N-MC and NS20Y, respectively. To investigate the function of this region, the intron and 245 bp at the 5'-end of exon 2 were investigated. Transient expression analyses using various chloramphenicol acetyltransferase constructs showed that the transcriptional activity of the intron is higher than that of the upstream promoter by 12-fold in SK-N-MC cells and by 5.5-fold in NS20Y cells in an orientation-dependent manner, indicating that the D1A intron is a strong promoter. Primer extension and ribonuclease protection assays revealed that transcription driven by the intron promoter is initiated at the junction of intron and exon 2 and at a cluster of nucleotides located 50 bp downstream from this junction. The same transcription start sites are utilized by the chloramphenicol acetyltransferase constructs employed in transfections as well as by the D1A gene expressed within the human cDNA. The relative abundance of D1A transcripts originating from the upstream promoter compared with those transcribed from the intron promoter is 1.5-2.9 times in SK-N-MC cells and 2 times in the human cDNA. Transcript stability studies in SK-N-MC cells revealed that longer D1A mRNA molecules containing exon 1 are degraded 1.8 times faster than shorter transcripts lacking exon 1. Although gel mobility shift assay could not detect DNA-protein interaction at the D1A intron, competitive co-transfection using the intron as competitor confirmed the presence of trans-acting factors at the intron. These data taken together indicate that the human D1A gene has two functional TATA-less promoters, both in D1A expressing cultured neuroblastoma cells and in the human striatum.

Dopamine mediates important brain functions including the generation of coordinated motor output, neuroendocrine modulation, behavior, memory, and cognition (1, 2). The receptors subserving the dopamine signaling pathways belong to the large family of seven-transmembrane, G-protein-coupled receptors (3). Five of these dopamine receptor genes have thus far been identified and classified into two main subfamilies: the D1 class, which includes the D1A and D1B (D5) receptors, and the D2 class, which includes D2, D3, and D4 receptors. (4-11).

Based largely on clinical pharmacologic observations in Parkinsonian and schizophrenic patients, the D2 dopamine receptor has traditionally been thought to be the main mediator of the motor and behavioral effects of dopamine (12, 13). More recent molecular and neurophysiologic advances in addition to the availability of receptor-selective pharmacologic agents have clarified the importance of the D1 class of dopamine receptors as well (14, 15). The D1A receptor is one of two dopamine receptors abundantly expressed in the striatum (16), suggesting that it has a critical role in transmitting the nigrostriatal dopaminergic signal resulting in normal motor function. In the prefrontal cortex, the D1A receptor is more abundantly expressed than the D2 receptor (17) and has recently been shown to modulate memory (18).

Information about the molecular regulation of this receptor would help us understand how it could be modified by disease states and by pharmacologic manipulations. To study transcription control of the human D1A dopamine receptor gene, we had previously cloned and characterized its 5'-flanking region and analyzed its main activator (19, 20). We found that this gene has a short noncoding exon 1 of about 450 bp, a 116-bp intron, and a long coding exon 2 (see Fig. 1). Thus, the 5'-noncoding region of the D1A gene is about 920 bp long. In the present investigation, we discovered that in addition to its original TATA-less promoter upstream of exon 1, this gene is also transcribed from a second strong promoter located in its intron, generating shorter transcripts lacking exon 1.

MATERIALS AND METHODS

Plasmid Constructions—Plasmids pCAT-D1–1102/525 and pCAT-D1–1102/708 were constructed from pCAT-D1–1102 (20) by deleting the respective fragments using appropriate restriction enzymes and religation (Fig. 2). The human D1A upstream promoter region (D1Pro, bases 1–963 relative to the first ATG) was generated by PCR using pCAT-D1–1102 as template with sense primer PP-1, 5'-CCCCAAGCTTTCCGCGGGAACCCCGCCGGCC-3' (inserted HindIII site underlined), and antisense primer PP-2, 5'-AAGCTTGCAGCTCGGGCGGCCTTCCAGGCTCT-3' (inserted PstI site underlined). The resultant fragment was digested with HindIII and PstI and inserted into the corresponding sites of pCAT-Basic (Promega, Madison, WI) (yielding pCAT-D1Pro) and of pCAT-Enhancer (Promega) (yielding pCAT-SVEnh-D1Pro). A similar strategy was employed to subclone the intron (nucleotides −599 to −484) between the PstI-XbaI sites of the same two vectors generating

1 The abbreviations used are: bp, base pair(s); CAT, chloramphenicol acetyltransferase; RPA: ribonuclease protection assay; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) M85247.

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Transfections were carried out using the CaPO\(_4\) co-precipitation method, using equal amounts of the test pCAT-\( \beta \)-\( \text{GAL} \) (233) 5 \( \mu \)g each for NS20Y and SK-N-MC cells and 10 \( \mu \)g each for HepG2 cells per 100-mm culture dish. For competitive co-transfections, SK-N-MC cells were co-transfected with 3 \( \mu \)g of pCAT-D1Int (+), 2 \( \mu \)g of pCMV-\( \beta \)-\( \text{GAL} \) (Clontech Laboratories, Inc., Palo Alto, CA), and 8 or 16 \( \mu \)g of the competitor plasmid pZero-D1Int (molar ratio to the test pCAT plasmid 1:4 and 1:9, respectively) supplemented with pZero to make up a total of 20 \( \mu \)g of DNA/plate. All plasmids used in transfections were purified by Plasmid Maxi Kit (Qiagen, Inc., Chatsworth, CA). Lysates were prepared by four cycles of freezing and thawing of the harvested cells followed by centrifugation.

CAT assays were carried out using the CAT enzyme-linked immunosorbent assay kit (Boehringer Mannheim). All CAT assay results were normalized to \( \beta \)-galactosidase activity (24).

### Primer Extension—Total RNA was extracted from SK-N-MC cells using RNAzol B (Tel-Test, Inc., Friendswood, TX), and primer extension was carried out using the Primer Extension System (Promega).

An oligodeoxynucleotide complementary to nucleotides 413 to 384 in exon 2 (5'-TCTGGAGGTGAGGACAAGTTTCCCTC-3') (Fig. 1) was 5'-end-labeled with \( \gamma \)-\(^{32}\)P-ATP using T4 polynucleotide kinase (24). Thirty-one \( \mu \)g of total RNA from SK-N-MC cells and 0.1 pmol of labeled primer were mixed in the presence of 2 \( \times \) primer extension buffer and annealed at 85°C for 10 min followed by cooling to room temperature for 10 min. The annealed primer was extended with avian myeloblastosis virus reverse transcriptase at 42°C for 30 min and ethanol-precipitated. The sample was resuspended in 5 \( \mu \)l of gel loading dye, separated in a denaturing 8\% urea, 6\% polyacrylamide gel.

### Gel Mobility Shift Assays—RNA was extracted and processed for RPA as described above. The relative densities of the protected bands were measured using a PhosphoImager model 445Si (Molecular Dynamics, Sunnyvale, CA), or the autoradiogram was subjected to densitometric analysis using a CO viz. (77, Sony) and NC-Image (1000) (10).

### Measurement of mRNA Stability—SK-N-MC cells were treated with actinomycin D (10 \( \mu \)g/ml; Sigma) in fresh medium for various durations. At the end of each treatment period, cells were harvested, and total RNA was extracted and processed for RPA as described above. The relative densities of the protected bands were measured using a PhosphoImager model 445Si (Molecular Dynamics).

#### RESULTS

**Characterization of the Downstream Promoter in the Human \( \text{DA} \) Gene Intron—Serial 3' deletions of the 5'-noncoding region of the human \( \text{DA} \) dopamine receptor gene, including the intron and 5'-end of exon 2, resulted in a dramatic decrease in transcriptional activity of the reporter gene in two \( \text{DA} \)-expressing neuroblastoma cell lines, SK-N-MC and NS20Y (Fig. 2). Approximately 80% of CAT activity was lost by removing the region between nucleotides 525 and \( -339 \) in SK-N-MC cells and about 40% in NS20Y cells. In the negative control cell line HepG2, where the \( \text{DA} \) gene is transcriptionally silent, a mod-
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The distinct localization of a transcription stimulator in the D_{IA} intron raised the possibility that this region could function as an independent promoter. To confirm this hypothesis, additional CAT constructs were made by inserting the D_{IA} intron in both orientations into pCAT-Basic and pCAT-Enhancer both lacking any promoter sequences (Fig. 2). A marked increase in CAT activity with constructs having the intron in the original orientation was detected in all three cell types tested, indicating that the intron harbors strong promoter activity. Compared with the D_{IA} upstream promoter (pCAT-D1Pro), the intron (pCAT-D1Int(+)) was 12- and 5.5-fold more active in SK-N-MC and NS20Y cells, respectively. On the other hand, subcloning the intron in the 3’ → 5’ orientation resulted in dramatic loss of most of its transcriptional activity in all three cell types. Evaluation of constructs that included both the intron and the upstream promoter simultaneously in different orders showed that the transcriptional activity of pCAT-D1Pro-D1Int was weaker than that of pCAT-D1Int-D1Pro (Fig. 2).

Identification of Multiple Transcription Initiation Sites Driven by the Intron Promoter—Transcription start sites driven by the intron promoter were determined by primer extension analysis and by RPA. For primer extension, an oligonucleotide primer located in exon 2 was 5’-end-labeled, hybridized with total RNA from SK-N-MC cells, and extended by avian myeloblastoma virus reverse transcriptase. Multiple transcription initiation sites were identified using this method, a prominent one being at the junction of intron and exon 2 (Fig. 3). Another small cluster of transcription start sites located about 50 bp downstream into exon 2 was also seen on the autoradiogram (data not shown).

To verify the data obtained from primer extension, RPA was performed. 32P-Labeled antisense RNA, transcribed from pGEM-E1E2 lacking the intron sequence, was hybridized with total RNA from SK-N-MC, HepG2, and yeast cells and digested with RNase A and T_{1}. A fragment protected by SK-N-MC mRNA was detected at the junction of exon 1 and exon 2 (Fig. 4). Three additional bands clustered approximately 50 bp downstream of the junction of exon 1 and exon 2 in the riboprobe were protected by mRNA from this D_{IA}-expressing cell line (Fig. 4). The positions of these bands were identical to those obtained with primer extension. Transcription initiation by the upstream D_{IA} promoter was also detected by RPA (Fig. 4). No protected band was seen by either HepG2 or yeast RNA (Fig. 4A).

The relative abundance of transcripts originating from the two D_{IA} promoters was estimated by quantitating the densities of bands indicated by arrows in Fig. 4, A and B, using a

| CAT activity (Fold to pCAT-Basic) | SK-N-MC | NS20Y | HepG2 |
|----------------------------------|--------|-------|-------|
| pCAT-D1Pro                       | 2.1    | 3.0   | 2.9   |
| pCAT-D1Pro-D1Int                 | 14.8   | 15.9  | 1.3   |
| pCAT-D1Int-D1Pro                 | 27.1   | 24.0  | 4.5   |
| pCAT-D1Pro-Ex                    | 5.9    | 3.2   | 0.3   |
| pCAT-D1Int(+)                    | 38.8   | 16.9  | 1.0   |
| pCAT-D1Int(+)                    | 25.5   | 16.7  | 3.6   |
| pCAT-D1Int(+)                    | 3.2    | 2.6   | 0.9   |
| pCAT-SVEnh-D1Pro                 | 62.0   | 2.3   | 10.8  |
| pCAT-SVEnh-D1Int(+)              | 48.6   | 12.2  | 8.8   |
| pCAT-SVEnh-D1Int(-)              | 25.3   | 1.7   | 2.7   |
| pCAT-Enhancer                    | 38.4   | 1.6   | 4.7   |
| pCAT-Basic                       | 1.0    | 1.0   | 1.0   |

Fig. 2. Transient expression analyses of various CAT constructs of the human D_{IA} dopamine receptor gene. CAT expression plasmids were generated by inserting various portions of the D_{IA} gene into the promoterless plasmid pCAT-Basic or into pCAT-Enhancer. These plasmids were transfected into human neuroblastoma SK-N-MC, murine neuroblastoma NS20Y, and human hepatoblastoma HepG2 cell lines using the CaPO4 co-precipitation method. CAT activity was measured using the CAT enzyme-linked immunosorbent assay kit and normalized with β-galactosidase activity derived from pRAS-B-GAL uniformly co-transfected into each plate. The direction of the arrows indicates the orientation of the inserts in the constructs. 1 and 2 refer to the order of inserts both in the 5’ → 3’ orientation. Transfections were repeated at least 3 times, yielding reproducible results.

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| Restriction Enzyme | EcoRI | HindIII | SspI |
|--------------------|-------|---------|------|
| pCAT-Enhancer      | CAT   | CAT     | CAT  |
| pCAT-Basic         | CAT   | CAT     | CAT  |

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Table 1: Restriction Enzyme Digests of CAT Constructs

- **EcoRI**: Digests at the restriction site in the CAT probe.
- **HindIII**: Digests at the restriction site in the CAT promoter.
- **SspI**: Digests at the restriction site in the CAT intron.
PhosphorImager as well as densitometric analysis of autoradiograms. In SK-N-MC cells, the ratio between transcripts starting from the upstream promoter and those originating from the intron promoter was between 2.9 ± 0.3 (Fig. 4A) and 1.5 ± 0.3 (Fig. 4B).

Confirmation of Transcription Initiation Driven by the Intron Promoter in the Human Striatum—To prove that transcription initiation from the intron promoter of the D1A gene is also utilized in vivo, RPA was carried out with RNA isolated from the human brain (Fig. 5). A labeled antisense RNA probe, transcribed from pGEM-E1E2, was hybridized with total RNA from a human caudate tissue and digested with RNase A and T1. A parallel experiment using RNA from SK-N-MC cells was carried out as control. Both lanes showed a protected fragment of 150 bp corresponding to the junction of exon 1 and exon 2 and another protected band at approximately 50 bp downstream (Fig. 5).

Similar to the analysis in SK-N-MC cells (Fig. 4), quantitation of the relative abundance of D1A messages in the human caudate revealed that long transcripts containing exon 1 are 2.0 ± 0.2 times more prevalent than shorter transcripts lacking exon 1. Thus, steady-state levels of D1A messages transcribed from the two promoters appear to be equivalent in a cultured neuroblastoma cell line and in vivo.

Confirmation of the Same Transcription Start Sites Driven by the Intron Promoter in the CAT Constructs—Accurate initiation of transcription from the intron promoter was also confirmed in CAT construct-transfected cells. For transient transfection of SK-N-MC and NS20Y cells (Fig. 6), the 131-bp PstI-BsoFI fragment (from pCAT-D1Pro-All) that includes the entire intron and 15 bases from exon 2 was inserted upstream of the CAT gene in pCAT-Basic to yield pCAT-D1IntE. To generate antisense RNA probe, the 390-bp PstI-EcoRI fragments from pCAT-D1IntE, which includes the D1A intron, 15 bases of exon 2, and 259 bases from the CAT gene, was inserted into the corresponding sites of pGEM-3Zf(−) to yield pGEM-D1IntE. After transfecting SK-N-MC and NS20Y cells with pCAT-D1IntE, RNA was isolated and hybridized with 32P-labeled antisense RNA probe, transcribed from pGEM-D1IntE, and digested with RNase A and T1. These experiments indicated that the positions of transcription initiation sites utilized by the fusion construct corresponded to the same major start sites identified in RNA extracted from nontransfected SK-N-MC cells, namely the junction of D1A exons 1 and 2 in the riboprobe as well as a cluster located 50 bp downstream (Fig. 6).

Lack of Detectable Nuclear Protein Binding in the D1A Intron
Promoter by Gel Mobility Shift Assay—The presence of trans-acting factors interacting with the intron promoter was first addressed by gel mobility shift assays using nuclear extracts from SK-N-MC and NS20Y cells with PCR-generated DNA probes. D1Int, Ex, and All (Fig. 7). However, no detectable retarded band could be seen with a probe spanning the D1A intron using this method. On the other hand, using probes having the 5′-end of exon 2, probes Ex and All, two major retarded bands were seen with nuclear extracts from both cell lines, and the respective unlabeled DNA fragments could effectively compete away all these retarded bands. The difference in the gel shift pattern between the two cell lines points to their different molecular profiles, although both have an adequate transcription machinery to express the D1A gene. Other differences between these two cell lines were also noted in transient transfection assays (Fig. 2).

Competitive Co-transfection with the D1A Intron—To further investigate the presence of trans-acting factors at the intron promoter, competitive co-transfection assay was employed as an indirect evidence for functional DNA-protein interaction. SK-N-MC cells were co-transfected with pCAT-D1Int+ and the competitor plasmid pZero-D1Int, harboring the intron in an otherwise null vector. Only a 4-fold excess of the competitor plasmid compared with the test pCAT plasmid reduced transcriptional activity of the intron promoter by about 30%, and an 8-fold excess competitor reduced this activity by about 60% (p < 0.006) (Fig. 8).

Effect of the Activator Sequence, AR1, on the Upstream D1A Promoter and the Intron Promoter—We had previously found that the sequence between −1154 and −1136 (termed AR1) functions as a transcriptional activator for the human D1A dopamine receptor gene (20). To investigate whether AR1 activates the upstream D1A promoter or the downstream intron promoter, pCAT-D1Pro-AR1 and pCAT-D1Int-AR1 were constructed (Fig. 9A). Compared with the D1A upstream promoter alone, pCAT-D1Pro-AR1 was 2.9- and 2.5-fold more active in NS20Y and SK-N-MC cells, respectively (Fig. 9B). On the other hand, pCAT-D1Int-AR1 was less active in both cell types compared with the intron promoter alone (Fig. 9C). Thus, AR1 activates the upstream promoter and tends to repress the intron promoter of the human D1A dopamine receptor gene.

Relative Stability of Human D1A Transcripts—The half-lives of mRNA transcribed from each promoter of the human D1A dopamine receptor gene were measured by RPA using RNA from SK-N-MC cells treated with the RNA polymerase inhibitor actinomycin D for various time points (Fig. 10). Transcripts originating from the upstream D1A promoter decayed with a half-life of 1 h, whereas mRNA starting from the intron promoter degraded with a half-life of 1.8 h.

DISCUSSION

The D1A dopamine receptor gene consists of two exons, an upstream noncoding exon 1 of about 450 bp and a longer exon 2 where the entire receptor protein is encoded (Fig. 1). These two exons are separated by a small intron of 116 bp in humans (19), 115 bp in rats (28), and 97 bp in pigs (29). Previously, several lines of evidence localized a strong promoter just upstream of exon 1 in both the human and the rat D1A genes (19, 20, 28). The present studies revealed that deletion of the intron and 5′-end of exon 2 results in a dramatic decrease in transcription from the upstream promoter in two D1A-expressing neuroblastoma cell lines (Fig. 2). To investigate this phenomenon further, we subcloned these portions of the human D1A gene alone as well as with the upstream promoter in the absence of exon 1, since the latter has several consensus sequences for known transcription factors (20). Transient expression assays indicated that the intron in this gene harbors strong transcriptional activity with typical features of a promoter including its ability to induce reporter gene expression as well as its ability to be trans-activated by the heterologous SV40 enhancer, both in an orientation-dependent manner. Thus, the human D1A dopamine receptor gene is transcribed from two distinct promoters not only in cultured neuroblastoma cells but also in the human caudate, as evidenced by the
presence of shorter D1A transcripts lacking exon 1 in this brain region. To date, a large number of genes in various species have been discovered to have more than one promoter, including the genes coding for dystrophin (30–33), brain-derived neurotrophic factor (34, 35), acetyl-CoA synthetase (36), acetylcholinesterase (37), β1-integrin (38), growth hormone (39), growth hormone-releasing hormone (40), protein tyrosine kinase (blk) (41), and the A1 adenosine receptor (42).

Evaluation of the relative activity of the two D1A promoters indicated that the downstream promoter located in the intron is 12-fold stronger than the upstream promoter in SK-N-MC cells and 5.5-fold stronger in NS20Y cells. Interestingly, the combination of the two D1A promoters resulted in a transcrip-tional activity lower than the strongest intron promoter alone in SK-N-MC cells. Furthermore, switching the order of these two promoters in the reporter gene construct significantly changed transcription efficiency such that the presence of the intron 5'-of the D1A upstream promoter (pCAT-D1Int-D1Pro) rather than 3'- (pCAT-D1Pro-D1Int) regained all the activity of the intron. These observations suggest that there is a functional interaction between these two promoters in D1A-expressing neuroblastoma cell lines and that this interaction is dependent on their relative position.

Transcription initiation from the intron promoter in the human D1A gene is very consistent and faithful. All methods used showed that transcription driven from this promoter begins at the border between the intron and exon 2 and at a cluster of nucleotides located 50 bp downstream. The positions of transcription start sites driven by the intron promoter are also preserved faithfully in the CAT fusion gene. This observation indicates that these start sites are utilized regardless of the sequence following the promoter and that they are indeed driven by the intron rather than the upstream promoter, since the latter was absent from the CAT construct (Fig. 6). More importantly, the endogenous D1A gene expressed in the human caudate has transcripts initiated from the original upstream promoter as well as from the intron promoter (Fig. 5).

The upstream promoter in the D1A gene is TATA-less and is GC-rich with multiple consensus sequences for Sp1 binding (19, 20), features seen in many housekeeping as well as regulated genes (43–49). The D1A intron promoter also lacks a TATA box and a CAAT box, but it is not GC-rich, and there are no consensus sequences for Sp1 binding. Although gel shift could not identify DNA-protein interactions at the intron (Fig. 7), competitive co-transfection clearly gave indirect evidence for the presence of such trans-acting factors binding to the

Fig. 7. Gel mobility shift assay with DNA probes, D1Int (the entire intron), Ex (245 bases of exon 2), and All (the entire intron and 245 bases of exon 2) (see Fig. 1). DNA probes were generated by PCR using 32P-labeled primers for one end and cold primers for the other end using pCAT-D1–1102 as template. About 5 fmol (>50,000 cpm) of probe and 5 μg of nuclear extract from SK-N-MC cells or 3 μg from NS20Y cells were used. All probes were purified by PAGE. Competitors indicate the unlabeled fragments corresponding to each probe. Competitors were added in 100- or 200-fold molar excess relative to the respective probes. Arrows indicate specific shifted bands seen in both cell lines.

Fig. 8. Competitive co-transfection. SK-N-MC cells were co-transfected with 3 μg of pCAT-D1Int (+) (see Fig. 2), 2 μg of pCMV-β-GAL, and 8 or 16 μg of the competitor plasmid pZero-D1Int (molar ratio to the test pCAT plasmid 1:4 and 1:8, respectively) supplemented with pZero to make up a total of 21 μg of DNA/plate. CAT activity of the test plasmid was measured using the CAT enzyme-linked immunosorbent assay kit and normalized with β-galactosidase activity. Means ± S.E. for four plates are shown as -fold compared with no competitor. *, analysis of variance, p < 0.006 compared with no competitor. This experiment was repeated twice with reproducible results.
intron promoter with high affinity, since even a small excess of competitor resulted in a significant functional impact (Fig. 8). Lack of detectable DNA-protein interaction at the D1A intron by gel mobility shift assay is consistent with previous reports demonstrating the difficulty in detecting nuclear factor binding at TATA-less promoters (50, 51). Nuclear factor binding, however, could be detected by gel shift with a probe in the 5' end of exon 2. The latter could be important in regulating the expression of the human D1A gene.

The presence of the intron promoter in the human D1A gene with the consequent generation of shorter transcripts lacking exon 1 adds to the complexity of molecular mechanisms involved in its expression. Comparison of the relative amounts of different D1A transcripts indicated that the longer transcripts with exon 1 are more abundant at steady state than the shorter versions in native SK-N-MC cells (Figs. 4 and 5) and in the human caudate (Fig. 5). Quantitatively, long D1A transcripts are 1.5–2.9 times more abundant and 1.8 times less stable than shorter transcripts in SK-N-MC cells (Figs. 4A and 10). Similarly, in the human caudate, D1A transcripts originating from the upstream promoter are 2 times more abundant than those initiated from the intron promoter. Thus, the relative activity of the two D1A gene promoters appear to be equivalent in a cultured neuroblastoma cell line and in striatal neurons in vivo. Although transfection experiments showed that the intron promoter alone is transcriptionally more active than the upstream D1A promoter in the two neuroblastoma cell lines tested, their presumed functional interaction in vivo may underlie the higher abundance of transcripts originating from the upstream promoter. In the presence of both promoters, the upstream promoter might be the predominant point where transcription is initiated preferentially. Although transient expression studies showed much higher CAT activity from constructs generating shorter transcripts lacking exon 1, the fate of the D1A message itself in cell lines and in the human caudate might be different than that of CAT transcripts.

The two promoters of the D1A dopamine receptor gene appear to be subject to regulation by different trans-acting factors, accounting for differences in their transcript abundance.

**Fig. 9. Transient expression analyses of the activator region, AR1, in CAT constructs containing the upstream promoter or the intron promoter.** A, schematic structure of CAT constructs. These plasmids were used to transfect the murine neuroblastoma NS20Y and the human neuroblastoma SK-N-MC cell lines using the CaPO4 co-precipitation method. CAT activity was measured using the CAT enzyme-linked immunosorbent assay kit and normalized with β-galactosidase activity derived from pRAS-β-GAL uniformly co-transfected into each plate. Means ± S.E. for three plates are shown as -fold compared with the respective promoter alone. B, *, analysis of variance, p < 0.0001 compared with pCAT-D1Pro. C, *, analysis of variance, p < 0.004 compared with pCAT-D1Int(+). These experiments were repeated twice with reproducible results.

**Fig. 10. Stability of human D1A transcripts.** Total RNA was extracted from SK-N-MC cells at the indicated times after treatment with actinomycin D (10 μg/ml). Labeled antisense RNA probe transcribed from a D1A cDNA that excludes the intron (pGEM-E1E2) was hybridized with 60 μg of total RNA, and the density of bands was quantitated by a PhosphorImager. The sum of the two major bands protected by RNA transcribed from the intron promoter (shown in Fig. 4A) is compared with the band protected by RNA transcribed from the D1A upstream promoter.

The presence of the intron promoter in the human D1A gene with the consequent generation of shorter transcripts lacking exon 1 adds to the complexity of molecular mechanisms involved in its expression. Comparison of the relative amounts of different D1A transcripts indicated that the longer transcripts with exon 1 are more abundant at steady state than the shorter versions in native SK-N-MC cells (Figs. 4 and 5) and in the human caudate (Fig. 5). Quantitatively, long D1A transcripts are 1.5–2.9 times more abundant and 1.8 times less stable than shorter transcripts in SK-N-MC cells (Figs. 4A and 10). Similarly, in the human caudate, D1A transcripts originating from the upstream promoter are 2 times more abundant than those initiated from the intron promoter. Thus, the relative activity of the two D1A gene promoters appear to be equivalent in a cultured neuroblastoma cell line and in striatal neurons in vivo. Although transfection experiments showed that the intron promoter alone is transcriptionally more active than the upstream D1A promoter in the two neuroblastoma cell lines tested, their presumed functional interaction in vivo may underlie the higher abundance of transcripts originating from the upstream promoter. In the presence of both promoters, the upstream promoter might be the predominant point where transcription is initiated preferentially. Although transient expression studies showed much higher CAT activity from constructs generating shorter transcripts lacking exon 1, the fate of the D1A message itself in cell lines and in the human caudate might be different than that of CAT transcripts.

The two promoters of the D1A dopamine receptor gene appear to be subject to regulation by different trans-acting factors, accounting for differences in their transcript abundance. For
instance, the activator sequence, AR1, activates the upstream promoter but tends to repress the intron promoter (Fig. 9). Moreover, the very long (about 920 bp) 5'-noncoding portion of the human DLA gene message may play an important role in modulating its translation analogous to the rat insulin-like growth factor message (52, 53) and the human transferrin and ferritin messages (54, 55). Whether the two types of DLA transcripts are translated at different efficiencies remains to be investigated. Since exon 1 in this gene is noncoding, the resultant receptor protein is no different whether translated from the short or long transcripts. The conserved structural organization of the DLA gene among rats, pigs, and humans (19, 28, 29) might suggest evolutionary significance of the intron and the resultant two different transcripts. The presence of two promoters may be required to ensure transcription of this important gene in the brain. Whether these two promoters are utilized in a tissue-specific manner or activated differentially during ontogeny remains to be determined.

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