The Journal of Neuroscience, March 1994, 14(3): 1766–1774

Effects of Intrahypothalamic Administration of Antisense DNA for Progesterone Receptor mRNA on Reproductive Behavior and Progesterone Receptor Immunoreactivity in Female Rat

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Since reproductive behaviors of female rats can be correlated with estrogen-induced increases in progestin binding by hypothalamic neurons, we hypothesized that specific progesterone receptor (PR) antisense DNA sequences might decrease these behaviors. Antisense oligonucleotides (15 bases), spanning the translation start site of rabbit PR mRNA, were microinjected directly among ventromedial hypothalamic neurons, and their behavioral effects were compared to control oligonucleotides composed of the same nucleotide bases in scrambled order. When applied 12 but not 24 hr after estradiol, the PR antisense treatment significantly reduced lordosis behavior, measured either as a reflex or in a mating behavior test. Notably, proceptive behaviors, which are strongly progesterone dependent, were greatly reduced in their occurrence (80% decrease). To see if PR protein was also reduced, antisense DNA was administered near the ventromedial hypothalamus on one side of the brain, while the other side received the scrambled control sequence or vehicle. The total number of PR-immunoreactive cells on the antisense side was significantly lower in the ventromedial nucleus, but not in control measurements from the medial preoptic area. Interrupting gene expression for PR, a transcription factor, in hypothalamic neurons, can have behavioral and immunocytochemical effects.

[Key words: antisense DNA, progesterone receptor, lordosis, proceptive behavior, progesterone receptor immunoreactivity, estrogen, ventromedial nucleus, arcuate nucleus, medial preoptic area]

Cloning and sequencing of genes expressed in the nervous system, followed by expression and regulation studies, provide molecular measurements whose possible connections to nervous system function are only correlational. Active manipulation of gene expression pathways can prove the importance of a given molecular event for a specific behavior.

The progesterone receptor (PR) is a transcription factor now cloned and analyzed extensively (Loosfelt et al., 1986; Conneely et al., 1987; Gronemeyer et al., 1987; Misrahi et al., 1987, 1988; Kastner et al., 1990; Schott et al., 1991). Expressed in brain (Kato and Onouchi, 1977; MacLusky and McEwen, 1980; Parsons et al., 1982), its mRNA is regulated by estradiol in a tissue-specific manner (Romano et al., 1989; Bayliss and Millhorn, 1991) in genetic females but not males (Lauber et al., 1991). We hypothesized that if PR mRNA increases correlate with facilitation of reproductive behaviors and actively drive them, then effective PR antisense sequences might reduce those behaviors. We tested this hypothesis with lordosis behavior, the circuitry of which is well known (Pfaff and Schwartz-Giblin, 1988) and whose correlation with PR is established (Moguilewsky and Raynaud, 1979; Parsons et al., 1980), but, more importantly, tested with proceptive behaviors for which progesterone dependence is even more pronounced (Hardy and DeBold, 1971; Whalen, 1974). In the present study we treated animals with oligonucleotides 12 hr after estrogen injection in order to maximize possible behavioral effects of intracerebrally administered antisense DNA, since a previous *in situ* hybridization study has shown that PR mRNA induction by estrogen is already at peak by 24 hr (Romano et al., 1989).

It is now well established that antisense DNA can effectively change expression of targeted genes in various *in vitro* systems (for reviews, see Uhlmann and Peyman, 1990; Cohen, 1991; Mirabelli et al., 1991). Although mechanisms of antisense DNA actions on neuronal cells *in vivo* are not currently known, it is important to determine whether antisense DNA administered directly into the brain could actually affect a functional endpoint. Therefore, we examined PR immunoreactivity in ventromedial hypothalamic neurons to assess changes in PR protein levels that may parallel behavioral effects of antisense DNA mentioned above. The immunocytochemical method was chosen because (1) it provides anatomical resolution of PR-positive cells (i.e., in comparison to biochemical PR binding assay), (2) animals can be treated very similarly to those used for behavioral study (i.e., in comparison to PR autoradiography in which *in vivo* injection of radioactive ligand is required), and (3) estrogen-inducible changes are well characterized (Warembourg et al., 1986; Blaustein et al., 1988; Blaustein and Turcotte, 1989; DonCarlos et al., 1989).

The purposes of this study can be summarized as follows: (1) to assess the behavioral effects of local administration of antisense sequence for PR mRNA on female sexual behaviors, specifically, to analyze which behaviors are most affected and to determine whether the effects are time dependent after estrogen injection; and (2) to determine whether progesterone receptor protein is actually reduced by PR antisense DNA.

Some of the results have been reported in preliminary form (Ogawa et al., 1992a,b).

Received June 9, 1993; revised Aug. 11, 1993; accepted Sept. 9, 1993.

We gratefully acknowledge the gift of antibody LET 548 from Dr. E. Milgrom, assistance with data analysis by Dr. C. Pavlides, and helpful discussions with Dr. M. M. McCarthy. This work was supported by NIH Grant HD-05751.

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Materials and Methods

Preparation of oligonucleotides

Unmodified phosphodiester oligonucleotides were used in the present study. Antisense oligonucleotides (15 bases) were chosen to span the translation start site of rabbit progesterone receptor mRNA (Fig. 1). Control oligonucleotides were composed of the same nucleotide bases in scrambled order. Avoidance of possible cross-hybridization with other rodent gene sequences was confirmed using a computer-based gene sequence search program (FASTA). Both antisense and scrambled control oligonucleotides were synthesized by Oligos Etc. Inc. (Wilsonville, OR) and further purified before use.

Antisense Oligonucleotides	5'-	CTC	AGT	CAT	GTC	GAC	-3'
PR mRNA	3'-	GAG	UCA	GUA	CAG	CUG	-5'
Scrambled Control Oligonucleotides	5'-	TGA	CGT	ATC	ACC	GTC	-3'

Figure 1. Sequences of antisense and scrambled sequence control oligonucleotides. Antisense oligonucleotide (15 bases) was chosen to span the translation start site (*underlined*) of rabbit PR mRNA nucleotide sequence. Control oligonucleotide was composed of the same nucleotide bases in scrambled order, without homology to other rat genes.

Animals

A total of 28 ovariectomized female Sprague–Dawley rats (200–250 gm), purchased from the Charles River Laboratories, were used. Twelve animals were used each for the behavioral and immunocytochemical studies, and four animals were used to determine the spread of oligonucleotide. Male intact Sprague–Dawley rats (300–350 gm), also purchased from the Charles River Laboratories, were used for mating behavior tests. All animals were maintained on a 12:12 hr light/dark cycle, lights off at noon, with constant temperature (25°C). Food and water were available ad libitum.

Behavioral study

Cannula implantation and screening tests. Animals were anesthetized with Chloropent (1 mg/10 gm, i.p.) and implanted with bilateral 22 gauge guide cannulas directed at the ventromedial nucleus of the hypothalamus (VMN): AP, bregma −3.1 mm; ML, 0.5 mm; depth, −9.0 mm. One week following postsurgery recovery, they were screened for 2 weeks for sexual receptivity with estradiol benzoate (EB; 1 or 10 μg) plus progesterone (P; 500 μg) priming. Females that failed to show any lordosis behavior in both tests were eliminated from the study.

Behavioral testing procedure. In each experiment, females (N = 12) were treated in the following sequence: (1) injected subcutaneously with 1 μg of estradiol benzoate (EB) at 0 hr, (2) microinjected bilaterally (over 90 sec) into the VMN through 28 gauge infusion cannulas with antisense or scrambled control oligonucleotides (400 ng dissolved in 500 nl of sesame oil for each side) at 12 hr (12 hr test) or 24 hr (24 hr test) under light Metofane anesthesia, and (3) injected subcutaneously with 500 μg of progesterone (P) at 44 hr (4 hr before behavioral tests). Starting at 48 hr after EB injections, females were tested in two different behavioral paradigms.

(1) Mating behavior tests: females were tested to measure mating behavior with an intact male rat in a testing chamber (92 × 24.5 cm) under dim red light during the dark portion of the light : dark cycle. Each test lasted until the female had received 15 mounts by the male. For each mount, the following behaviors were recorded: (1) occurrence of lordosis, (2) occurrence of preceding proceptive behavior (hopping or darting), (3) occurrence of preceding rejection behavior (kicking, biting, or lunge), and (4) occurrence of preceding vocalization. For each female, the following ratios were calculated: (1) lordosis quotient = number of lordoses/number of mounts × 100, (2) proceptive behavior ratio = number of mounts preceded by proceptive behavior/number of total mounts × 100, (3) rejection behavior ratio = number of mounts preceded by rejection behavior/number of total mounts × 100, and (4) vocalization ratio = number of mounts preceded by vocalization/number of total mounts × 100.

(2) Lordosis reflex tests: after the completion of mating tests, lordosis reflex intensity was assessed by applying manual cutaneous stimulation five times and rating it using a scale from 0 (no vertebral dorsiflexion) to 3 (strongest possible response) with 0.5 intervals. Rating of lordosis reflex intensity was performed by an experimenter who was blind with regard to the treatment of animals. The average of five ratings was calculated for each female.

Each rat served as its own control. Thus, each rat was tested four times, always 9–12 d apart. They were first tested following microinfusion of either antisense or scrambled control oligonucleotides, delivered at 12 hr after EB injections. Across rats, oligonucleotide infusions were counterbalanced for order. Animals were then studied with microinfusions of either antisense or scrambled control oligonucleotides, which were delivered at 24 hr after EB injections and counterbalanced for order across rats.

Histology. Following the completion of behavioral tests, animals were perfused and the brain sections were stained with cresyl violet for microscopic examination of cannula location.

Immunocytochemical study

Infusion of oligonucleotides. Ovariectomized female Sprague–Dawley rats (N = 12), different from those used in the behavioral study, were injected subcutaneously with either 2, 5, or 10 μg of EB. At 12 hr after EB, PR mRNA antisense oligonucleotides (400 ng dissolved in 250 nl of sesame oil) were administered in one side of the brain through a 30 gauge needle aimed dorsal to the VMN (AP, bregma −2.9 ~ −3.1 mm; ML, 0.07 mm; depth, −9.0 mm), under Chloropent anesthesia (1 mg/10 gm, i.p.). To make a precise comparison within each animal, the other side of the brain received either scrambled sequence control oligonucleotides or vehicle. Forty-eight to fifty hours after EB, the brains were processed for immunocytochemistry for progesterone receptor.

Progesterone receptor immunocytochemistry. Rats were deeply anesthetized and perfused transcardially with (1) 100 mM phosphate-buffered saline (PBS) containing 0.1% heparin, pH 7.2; (2) 4% paraformaldehyde in 400 mM phosphate buffer containing 100 mM lysine and 10 mM sodium-*m*-periodate, pH 7.2; and (3) PBS containing 10% sucrose. Brains were blocked and stored overnight at 4°C in PBS containing 30% sucrose. Tissue blocks containing the medial preoptic area and ventromedial and arcuate nuclei of the hypothalamus were cut at 30 μm on a freezing microtome. Free-floating sections were stored in 50 mM Tris-buffered saline (TBS), pH 7.2, at 4°C for 1–4 hr and washed for 10 min in TBS, pH 7.2, containing 0.5% Triton X-100 and 1.5% normal horse serum (Vector) at room temperature. They were then sequentially incubated in (1) a 1:3000 dilution of the anti-PR antibody, LET 548 (gift of Dr. E. Milgrom, Hôpital de Bicêtre) in TBS, pH 7.2, containing 0.5% Triton X-100 and 1.5% normal horse serum for 96–120 hr at 4°C; (2) a 1:200 dilution of the biotinylated horse anti-mouse secondary antibody (Vector) in TBS containing 0.5% Triton X-100 and 1% of normal horse serum (TBSHT) for 90 min at room temperature; and (3) the avidin-biotin-complex (Vectastain ABC Elite kit, Vector) in TBSHT for 40 min at room temperature. After each incubation period, sections were rinsed in TBS for 30 min (3 × 10 min) at room temperature. They were then treated with diaminobenzidine and 0.03% hydrogen peroxide in TBS, and staining was further intensified with 1% nickel-cobalt chloride. Control conditions involved omitting the primary antibody from the staining procedure.

Antibody. LET 548 is a monoclonal antibody raised in mouse, and recognizes the epitope located at amino acids 295–325 of the rabbit PR. LET 548 cross-reacts with rodent PR and has been extensively characterized (Lorenz et al., 1988; Groyer-Picard et al., 1990).

Histology. Every fifth section was stained with cresyl violet for microscopic examination of location of injection needles.

Spread of infused oligonucleotides

The oligonucleotides were 5'-end labeled with ^{32}P-ATP using T_4 polynucleotide kinase. The labeled oligonucleotides were then reconstituted in 500 nl of sesame oil and injected unilaterally with the method described in Immunocytochemical study, above. At either 12 (N = 1), 24 (N = 1), or 36 hr (N = 2), brains were removed and tissue blocks containing the medial preoptic area and ventromedial and arcuate nuclei of the hypothalamus were cut at 10 μm on a cryostat. All the sections were exposed to Kodak scientific imaging films. Spread of the labeled substance was determined by measuring optical density of autoradiograms with an image analysis system (IMAGE 1.43, NIH).

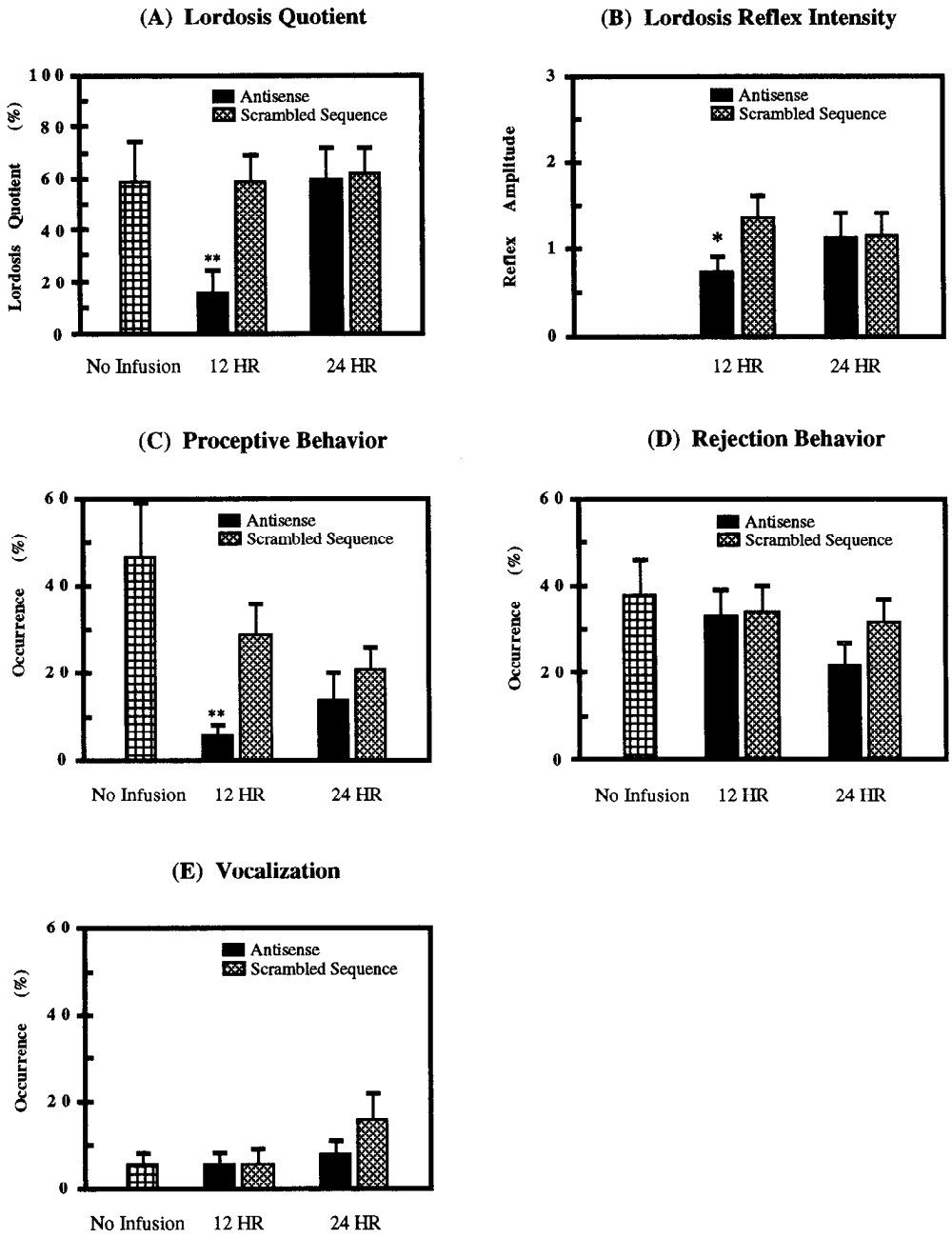

Figure 2. Effects (mean ± SEM, $N = 12$) of PR antisense DNA administration on lordosis behavior (number of lordoses/number of mounts × 100) during the mating behavior tests (*A*), lordosis reflex intensity during the lordosis reflex tests (*B*), proceptive behavior (number of mounts preceded by proceptive behaviors/number of total mounts × 100) (*C*), rejection behavior ratio (number of mounts preceded by rejection behavior/number of total mounts × 100) (*D*), and vocalization ratio (number of mounts preceded by vocalization/number of total mounts × 100) (*E*), in the mating behavior tests. *, $p < 0.05$; **, $p < 0.01$ (*t* test). *No infusion,* data from the screening test; *12HR* and *24 HR,* 12 hr test and 24 hr test in which oligonucleotides were infused 12 hr or 24 hr after estrogen injection, respectively.

Results

Behavioral study

PR antisense DNA infusion greatly modified mating behaviors in a time-dependent and behaviorally specific manner (Fig. 2). The lordosis quotient measured in the mating behavior test (Fig. 2*A*) was decreased by 73% compared to control, following antisense DNA infusion at 12 hr after EB injection ($p < 0.01$). The lordosis reflex intensity assessed by applying manual cutaneous stimulation (Fig. 2*B*) was also reduced, by 44% compared to control ($p < 0.05$). Proceptive behavior (Fig. 2*C*) was greatly decreased, by 80% compared to control ($p < 0.01$). In contrast, rejection behaviors (Fig. 2*D*) and vocalization (Fig. 2*E*) were not affected. We also assessed the magnitude of lordosis responses in the mating behavior tests with a scale of 1 to 3. In contrast to lordosis quotient (a measure of frequency), magnitude (or amplitude) of lordosis (mean ± SEM) was not affected by antisense DNA (1.88 ± 0.31 for antisense DNA vs 2.21 ± 0.69 for scrambled sequence control).

Effects of antisense were also specific with respect to time of administration. Antisense given 24 hr after EB had no inhibitory effects on the lordosis quotient, lordosis reflex intensity, and proceptive behavior ratio.

Histological examination revealed that the tips of all the bilateral cannulae were located either just dorsal to the VMN or within the VMN (Fig. 3). Within the range of VMN sites, there was no correlation between infusion site and effectiveness of antisense DNA on reproductive behaviors.

Immunocytochemical study

PR-immunoreactive cells were detected within the VMN (most densely in the ventrolateral part of the nucleus), arcuate nucleus,

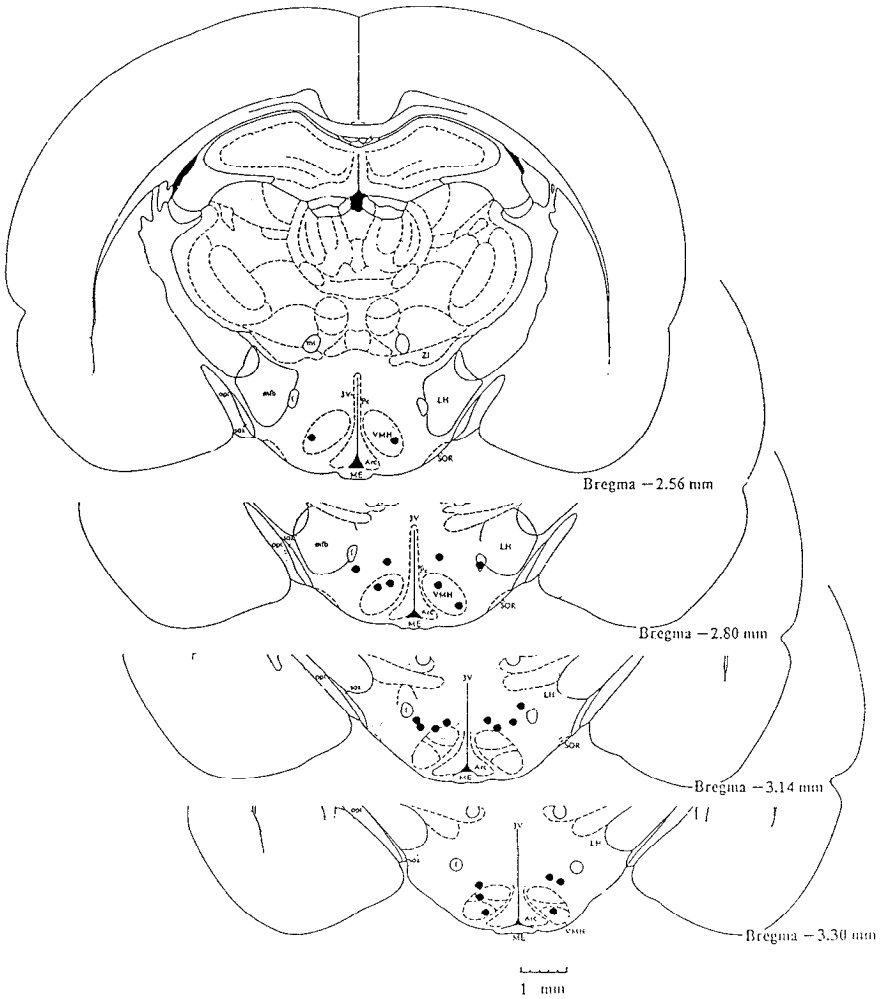

Figure 3. Histological diagrams (from Paxinos and Watson, 1986) depicting the location of bilateral infusion cannula tips in the hypothalamus in the behavioral study ($N = 12$). *3V,* third ventricle; *Arc,* arcuate nucleus; *f,* fornix; *LH,* lateral hypothalamus; *ME,* median eminence; *mfb,* medial forebrain bundle; *mt,* mammillothalamic tract; *opt,* optic tract; *Pe,* periventricular nucleus; *SOR,* supraoptic nucleus; *sox,* supraoptic decussation; *VMH,* ventromedial nucleus; *ZI,* zona incerta.

and the medial preoptic area, but not in cortex or amygdala. For each animal, five to eight sections (150 μm apart) containing the VMN and the arcuate nucleus were analyzed for PR immunoreactivity, and one section was analyzed for the medial preoptic area. The number of PR-immunoreactive cells on the side of brain injected with antisense oligonucleotide was compared to that on the side injected with scrambled sequence control oligonucleotide or vehicle control in each section. Absolute numbers of PR-immunoreactive cells/section (mean ± SEM) in the control infusion side of the brain were 83.96 ± 13.21 in the VMN, 25.22 ± 7.15 in the arcuate nucleus, and 95.08 ± 10.12 in the medial preoptic area. Control conditions involved omitting the primary antibody from the staining procedure and did not result in immunocytochemical staining.

PR immunostaining intensity as well as both darkly stained and the total number of PR-immunoreactive cells were decreased 48–50 hr after EB injection in the VMN on the side that received antisense DNA compared to the control infusion side (Figs. 4, 5; Table 1). The inhibitory effect of antisense DNA was detected throughout the anterior–posterior extent of the VMN, but was most obvious at the posterior end where more PR-immunoreactive cells were found (Figs. 4*D,* 5*A–D*). In the same brain sections, PR-immunoreactive cells were found in the arcuate nucleus (Fig. 4*A–D*), but the total number of PR-immunoreactive cells in the arcuate nucleus was much smaller than in the VMN and was not affected by injection of antisense DNA (Table 1). As a negative control, PR-immunoreactive cells were also counted in the medial preoptic area and did not change following the infusion (Table 1, Fig. 5*E*). Compared to 2 μg of EB, 5 or 10 μg of EB induced more PR-immunoreactive cells in the VMN of the control infusion side of the brain (mean numbers of PR-immunoreactive cells/section in the VMN were 30.14 with 2 μg EB, 88.83 with 5 μg EB, and 108.5 with 10 μg EB). However, there was no systematic relationship between the percentage of inhibitory effects of antisense DNA on PR immunoreactivity and the total number of PR-immunoreactive cells that had been induced by estrogen.

As shown in Figure 6, the tips of the injection needles were found just dorsal to the VMN at the same anterior–posterior level in both sides of the brain in all 12 animals analyzed. Therefore, when there was a failure of antisense oligonucleotide to inhibit PR immunoreactivity in individual animals, it was not due solely to the location of the injection needle.

Spread of infused oligonucleotides

Autoradiograms showed dense labeling in the area immediate to the tip of the needle (Fig. 7), while in the surrounding area labeling was much weaker. No labeling was detected in the contralateral side. It was found that ^{32}P-labeled oligonucleotides spread intensely about 500 μm along the rostrocaudal axis from the center of the injection needle tip. Between 500 and 1500 μm, the degree of oligonucleotide spread was diminished and

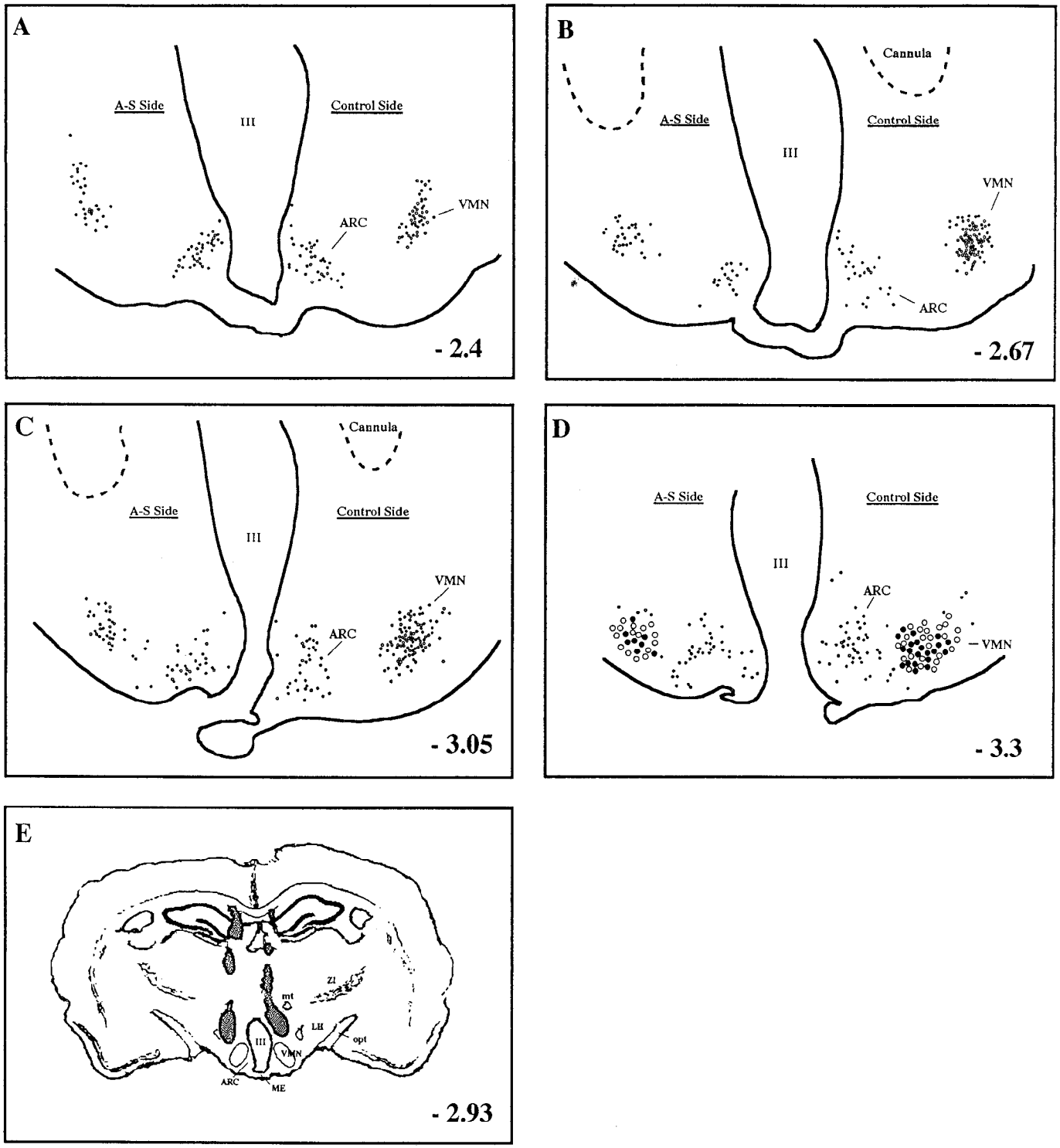

Figure 4. Schematic diagrams of hypothalamic tissue sections showing PR-immunoreactive cells in the VMN and arcuate nucleus from a representative animal in which antisense had inhibitory effects of average size (*A–D*). Each *small solid circle* represents a darkly stained cell, and each *small open circle* represents a lightly stained cell. In *D*, each *large solid circle* represents five darkly stained cells and each *large open circle* represents five lightly stained cells. The distance from bregma based on Paxinos and Watson (1986) is indicated on the *lower right corner* of each panel. Tracing of histology section (*E*) shows that the tips of injection needles were found just dorsal to the VMN at the same anterior–posterior level in both sides of the brain. *Shaded areas* depict needle tracks. *III,* third ventricle; *ARC,* arcuate nucleus; *f,* fornix; *LH,* lateral hypothalamus; *ME,* median eminence; *mfb,* medial forebrain bundle; *mt,* mammillothalamic tract; *opt,* optic tract; *VMN,* ventromedial nucleus; *ZI,* zona incerta.

beyond 1500 μm from the injection side, no isotope was detected. Thus, oligonucleotides infused toward the VMN spread in an area wide enough to cover the entire VMN (it extends about 1500 μm along the rostro–caudal axis) and locally enough not to affect the medial preoptic area.

Discussion

In the present study, it was found that intracerebral administration of antisense DNA for PR mRNA can have behavioral and immunocytochemical effects. Lordosis behavior was re-

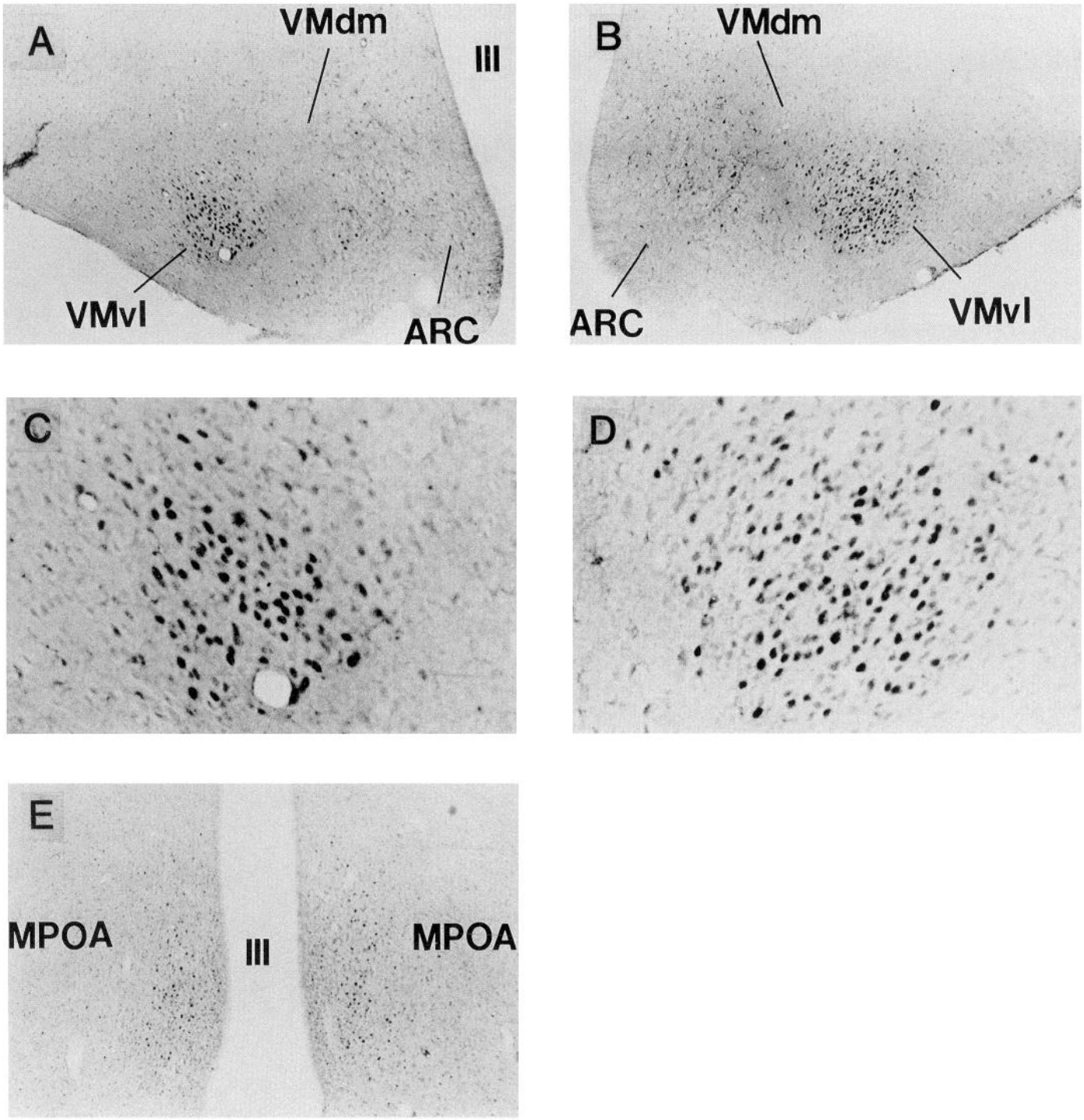

Figure 5. Photomicrographs of PR-immunoreactive cells in the VMN, arcuate nucleus, and medial preoptic area. The brain side infused with antisense oligonucleotides (*A*) had less PR-immunoreactive cells compared to the control (vehicle) infusion side (*B*) in the VMN but not in the arcuate nucleus. *C* and *D* are higher magnifications of *A* and *B*, respectively. There were 51 darkly stained cells and 85 lightly stained cells in the antisense side (*A* and *C*), whereas there were 100 darkly stained and 129 lightly stained cells in the control infusion side (*B* and *D*). There was no difference in number of PR-immunoreactive cells in the two sides of the brain (*right*, antisense infusion; *left*, vehicle infusion) in the medial preoptic area (*E*). The animal shown in *A–D* received 5 μg of EB and that in *E* received 10 μg of EB. *III*, third ventricle; *ARC*, arcuate nucleus; *MPOA*, medial preoptic area; *VMdm*, ventromedial nucleus, dorsomedial part; *VMvl*, ventromedial nucleus, ventrolateral part. Magnification: *A* and *B*, 75×; *C* and *D*, 200×; *E*, 60×.

duced whether measured by a mating behavior test or a lordosis reflex test. Furthermore, the effect of antisense DNA was most pronounced in proceptive behavior known to be strongly progesterone dependent. All these findings provide evidence for the importance of progesterone receptor gene expression in female reproductive behavior in rats. They also provide a molecular rationale for the effectiveness of the PR blocker RU486 in reducing lordosis behavior (Brown and Blaustein, 1984; Etgen and Barfield, 1986; Vathy et al., 1987). These behavioral effects of antisense DNA were likely due to its action on VMN neurons,

Table 1. Effects of antisense oligonucleotide infusion to VMN on number of PR-immunoreactive cells in three hypothalamic regions

VMN		ARC		MPOA	
Total cells	Darkly stained cells	Total cells	Darkly stained cells	Total cells	Darkly stained cells
0.75 ± 0.10*	0.62 ± 0.11**	1.10 ± 0.09	1.00 ± 0.11	1.04 ± 0.06	0.98 ± 0.07
Anterior half					
0.82 ± 0.12	0.77 ± 0.14*	1.28 ± 0.20	0.95 ± 0.14		
Posterior half					
0.74 ± 0.11*	0.59 ± 0.15*	1.00 ± 0.09	1.06 ± 0.21		

Data are presented as the ratio (mean ± SEM, $N = 12$) of number of PR-immunoreactive cells on the side of brain injected with antisense oligonucleotide to the side injected with scrambled sequence control oligonucleotide or vehicle control. Darkly stained cells were counted separately. For VMN and arcuate nucleus (ARC), the anterior half (bregma −2.4~−2.9) and posterior half (bregma −2.9~−3.4) of the nucleus were analyzed separately since most of PR-immunoreactive VMN cells were found in the posterior half. MPOA, medial preoptic area. *$p < 0.05$, **$p < 0.01$, Wilcoxon matched-pairs signed-rank test, one-tailed.

as very little spread of oligonucleotide was detected as far as another behaviorally relevant cell group, the medial preoptic area.

Effects of antisense were also specific with respect to time of administration. Antisense given 24 hr after EB had no inhibitory effects on any measurements (Fig. 2*A–C*). Previous studies in rat brain have shown that PR mRNA levels declined by 48 hr after the first EB injection even when a second EB injection was given at 24 hr (Romano et al., 1989) whereas maximum increases of hypothalamic PR levels were maintained between 24 hr and 72 hr after a single EB injection (Moguilewsky and Raynaud, 1979). In the present experiment, therefore, synthesis of PR for behaviorally relevant processes might be completed by 24 hr after EB. The behavioral effectiveness of PR antisense administration is consistent with results in breast cancer cell lines that estrogen-induced increases of the levels of PR protein are due to an increase in the rate of PR synthesis without altering the rate of PR degradation (Nardulli et al., 1988; Read et al., 1988).

PR-immunoreactive cells were induced by 2–10 μg of EB in the VMN, arcuate nucleus, and medial preoptic area but not in cortex or amygdala. This anatomical distribution was consistent with the previously reported distribution of PR-immunoreactive cells in EB-treated guinea pig brain (Warembourg et al., 1986; Blaustein et al., 1988; Blaustein and Burcotte, 1989; DonCarlos et al., 1989). Antisense DNA significantly decreased the number of PR-immunoreactive cells in the VMN. In the medial preoptic area, where systemically administered EB induced a substantial number of PR-immunoreactive cells but very little VMN-applied antisense oligonucleotide was detected,

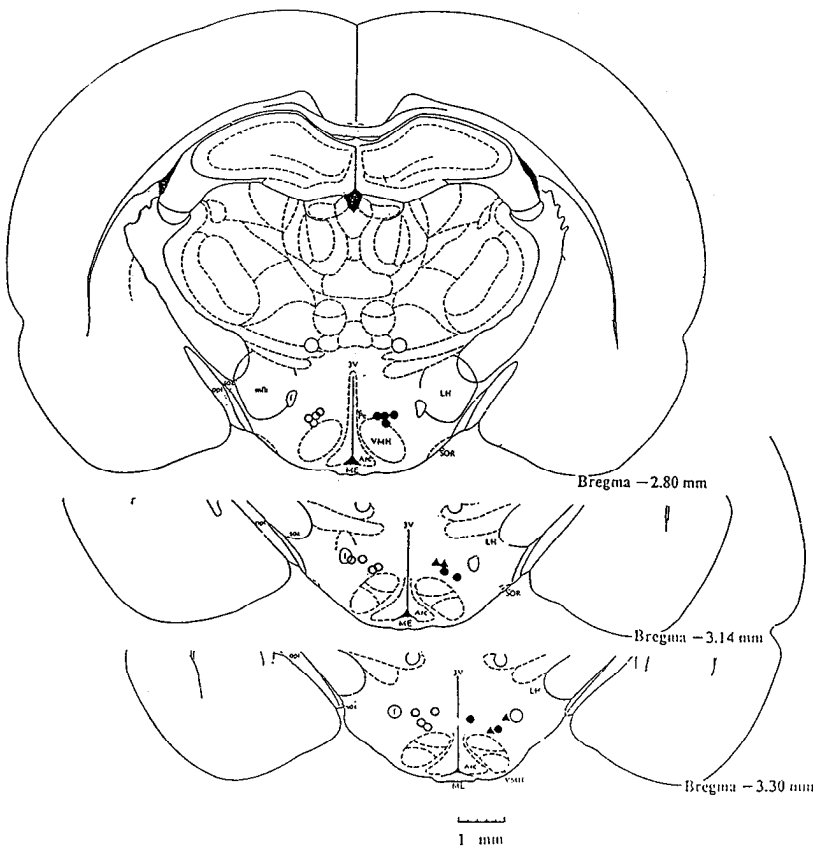

Figure 6. Schematic diagrams (from Paxinos and Watson, 1986) depicting the location of injection needle tips in the hypothalamus in the immunocytochemical study. To make a precise comparison in each animal, antisense DNA was infused on one side of the brain (*solid circle* or *triangle*) and either scrambled sequence control DNA or vehicle (*open circle*) was infused on the other side of the brain. *Circles* represent animals in which number of PR-immunoreactive cells in the antisense side was less than 80% of that in the control infusion side. *Triangles* represent animals in which antisense DNA had no effects on PR immunoreactivity. See Figure 3 for abbreviations.

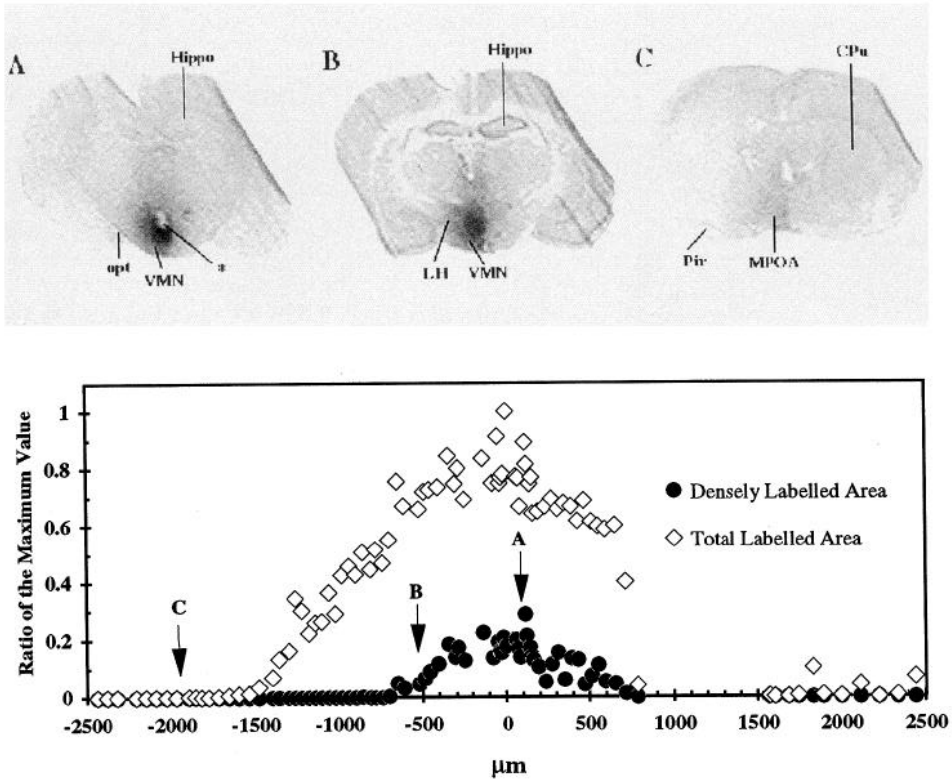

Figure 7. Spread of ^{32}P-labeled scrambled oligonucleotides injected into the left side of the brain. The brain was removed 36 hr after injection and processed as described in Materials and Methods. Three autoradiograms were taken from the injection site (*A*), 500 µm anterior to injection site (*B*), and 2000 µm anterior to the injection site (medial preoptic area region) (*C*). The *bottom panel* shows the spread of labeled substance along the rostrocaudal axis. In each brain section, the area with dense labeling (*solid circles*) detected around the tip of the injection needle was measured separately from the total labeled area (*open diamonds*). A densely labeled area was defined as an area in which the optical density was higher than the mean + four standard deviations higher than the entire brain section. The total labeling area included the area (in pixels) in which the optical density was more than two standard deviations away from the mean. All data were converted to a ratio of the maximum peak value. *Arrows* indicate sections corresponding to the autoradiograms. *Asterisk* (*), tip of the infusion needle; *CPu,* caudate putamen; *Hippo,* hippocampus; *LH,* lateral hypothalamus; *MPOA,* medial preoptic area; *opt,* optic tract; *Pir,* piriform cortex; *VMN,* ventromedial nucleus.

antisense DNA had no effect on PR immunoreactivity. Recently, we also have established that local administration of antisense DNA for PR mRNA aimed at the VMN can significantly reduce estrogen-induced ^{3}H-progestin binding in the basomedial hypothalamus but not in the medial preoptic area in mouse brain (Ogawa et al., 1993). These results further support the inference that PR protein levels can be manipulated by *in vivo* intracerebral application of antisense DNA for PR mRNA.

The overall magnitude of inhibitory action of antisense DNA on PR immunocytochemistry was, however, smaller than that found in the behavioral study. In the immunocytochemical study, injection of a higher dose of EB (2–10 µg) was needed to detect PR-immunoreactive cells instead of 1 µg used in the behavioral study, and it is possible that more PR mRNA was induced and was harder to block. Alternatively, behaviorally important actions of progesterone may be restricted to a small population of PR-immunoreactive cells.

To date, mechanisms of the behavioral effects of intracerebrally administered antisense DNA have not been specified. Translation arrest (Liebhaber et al., 1992) and RNA cleavage by RNase H (Furdon et al., 1989; Giles and Tidd, 1992) have been proposed as important steps, mainly based on studies of *in vitro* systems (for reviews, see Uhlmann and Peyman, 1990; Cohen, 1991). In *in vivo* application of antisense DNA, intracerebrally injected oligonucleotides are not well studied in contrast to intravenously or intraperitoneally injected oligonucleotides (Agrawal et al., 1991; Iversen, 1991; Zendegui et al., 1992). Moreover, although various types of modified oligonucleotides with different stability, efficiency of cellular uptake, and mechanisms of action are now available (Cook, 1991; Crooke, 1991; Stein et al., 1991; Zon and Geiser, 1991; Akhtar et al., 1992; Giles and Tidd, 1992), no systematic comparisons of effects on behavioral variables have been done. However, the findings of the present study as well as other reports about behavioral modification by antisense DNA in a variety of behavioral paradigms (McCarthy et al., 1991, 1993; Flanagan et al., 1992; Scarbrough and Wise, 1992; Wahlestedt et al., 1993) lead us to conclude that the antisense DNA strategy has a potential role in the study of the molecular bases of behavior.

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