Distribution of RNA Transcripts from Structural and Intervening Sequences of the Ovalbumin Gene

Abstract. *A study was made of the function of the intervening sequences in the ovalbumin gene. Radioactively labeled DNA probes for the intervening sequences were prepared and RNA's were isolated from whole cells, nuclei, and polysomes of estrogen-stimulated chick oviducts. The concentrations of messenger RNA (mRNA) transcripts from ovalbumin structural sequences ($mRNA_{ov}$) and transcripts corresponding to intervening sequences were then estimated by hybridization to cloned DNA probes. Oviduct tissue contains approximately 58,000 molecules of $mRNA_{ov}$ sequences per tubular gland cell and most of these sequences are present in the cytoplasm. In contrast, there are 200 to 300 molecules of RNA per cell which are transcribed from the intervening sequences of the natural ovalbumin gene and almost all of these are found in the nucleus. The difference in distribution of structural and intervening sequence transcripts suggests that, unlike mature mRNA, the intervening sequences are not preferentially transported to cytoplasmic polysomes.*

Regulation of ovalbumin gene expression in the chick oviduct is mediated by steroid hormones (*1–5*). By means of restriction endonuclease mapping and DNA sequencing it has been shown that the structural sequences of the natural ovalbumin gene and other eukaryote genes are interrupted by regions of nonstructural intervening sequences (*6–10*). The ovalbumin gene is divided into eight segments by seven intervening sequences (*10*). In an attempt to understand the functional importance of these intervening sequences and the processing of their messenger RNA (mRNA) transcripts, we have studied the relative accumulation in nucleus and cytoplasm of RNA corresponding to both the structural and intervening sequences within the ovalbumin gene.

The natural ovalbumin gene can be digested by restriction endonuclease Eco R1 into three major restriction fragments of 2.4, 1.8, and 9.5 kilobase pairs. These three fragments are arranged in sequence such that the 2.4-kb fragment codes for a 5′-portion and the 9.5-fragment codes for the 3′-end of mature ovalbumin mRNA ($mRNA_{ov}$). All of these pieces were cloned recently in our laboratory in plasmid pBR322 (*10, 11*). These fragments can be recovered in pure form (free of nonovalbumin gene sequences) from the clones, named pOV2.4 and pOV1.8, by Eco R1 digestion followed by gel electrophoresis (*12*). Since the third fragment, pOV9.5, contains mainly 3′-end flanking sequences and only a small part of the intervening sequences, it was not used in this study. After the purified fragments had been nicked with deoxyribonuclease, they were labeled with [^{3}H]dCTP (deoxycytidine triphosphate) and [^{3}H]TTP (thymidine triphosphate) by DNA polymerase I (nick translation) to specific activities of 6×10^7 and 4×10^7 counts per minute per microgram, respectively (*12*). These labeled fragments were then used as probes for intervening sequences without further purification since only 7 percent of the 2.4-kb fragment and 5 percent of the 1.8-kb fragment were protected from S1 nuclease digestion by hybridization with excess $mRNA_{ov}$. A pure single-stranded structural gene probe for $mRNA_{ov}$ sequences was prepared from complementary DNA (cDNA) clone pOV230 (*13*), which contains all of the structural sequences except 12 nucleotides at the 5′-end of the $mRNA_{ov}$. This probe lacked anticoding strand DNA sequences and host plasmid sequences. It was generated as described by Roop *et al.* (*12*).

These probes were then used to measure the concentration of RNA transcripts corresponding to structural and intervening sequences in total cellular RNA, nuclear RNA, and polysomal RNA of chick oviduct (*14*). The hybridization data obtained in these experiments are shown in Fig. 1. Complete (100 percent) hybridization was obtained for the structural gene probe and 45 percent or more was observed for the OV2.4 and OV1.8 probes. Since both strands of the ovalbumin gene were present in the OV2.4 and OV1.8 probes, the results are consistent with the view that only one of the DNA strands is transcribed into RNA in vivo. The low level of hybridization (around 10 percent) observed at low C_0t values (where C_0t is moles of nucleotide per liter times seconds) when the OV1.8 and OV2.4 probes were used can be attributed to hybridization of the small amount of structural sequences in these DNA probes to $mRNA_{ov}$ sequences (Fig. 1). Except at low C_0t values, single component hybridization curves were observed when the OV2.4 and OV1.8 probes were used. This result suggests that the transcripts of various intervening sequences within the 2.4 and 1.8 fragments exist at roughly equal concentration.

We have used the data in Fig. 1 to calculate the concentration of RNA transcripts representing structural and intervening sequences in different cellular compartments (Table 1). Oviduct tissue prepared from chicks stimulated with diethylstilbestrol (DES) contained approximately 58,000 molecules of $mRNA_{ov}$ sequences per tubular gland cell, which is similar to previously published values (*2–4*), and the majority of these sequences were found in the cytoplasm bound to polysomes. On the average, there were only 200 to 300 molecules of RNA per cell transcribed from the intervening sequences of the 2.4- and 1.8-kb fragment. In contrast, almost all of these transcripts were found in the nucleus.

Since the concentration of $mRNA_{ov}$ sequences in the cytoplasm was extremely high (40,000 to 50,000 molecules per tubular gland cell), it was important to demonstrate that the $mRNA_{ov}$ sequences measured in nuclei were not due to cytoplasmic contamination. Although it is difficult to completely rule out this possibility, we think that the contribution of cytoplasmic sequences to those measured in nuclei was minimal for the following reasons. First, the same amounts of $mRNA_{ov}$ were detected in RNA isolated from nuclei not treated with detergent as compared to nuclei that were repeatedly washed with 0.5 percent Triton X-100. Second, during secondary hormone stimulation of hormone-withdrawn chick oviduct, the kinetics of accumulation of $mRNA_{ov}$ in the nucleus and whole cell are dramatically different (*12*). After 48 hours of secondary stimulation, the concentration of $mRNA_{ov}$ per tubular gland cell nucleus reaches a plateau at 2500 to 3000 molecules, whereas that in the total cell continues to increase from 13,000 molecules at 48 hours of secondary stimulation to 40,000 to 50,000 at steady-state conditions. Third, the ovalbumin mRNA sequences observed in the nuclei are somewhat larger in size than those isolated from the cytoplasm when they are compared by analysis of denaturing agarose gels. This observation is consistent with the notion that nuclear $mRNA_{ov}$ sequences may contain a longer polyadenylate segment. Finally, and most important, the concentration of ovalbumin mRNA and ovomucoid mRNA ($mRNA_{om}$) sequences in isolated nuclei is very similar [2500 molecules of $mRNA_{ov}$, 2000 molecules of $mRNA_{om}$ (*15*)], whereas that observed in polysomal RNA or total cellular RNA is quite different (43,000 as opposed to 7000 in polysomal RNA and 60,000 as opposed to 9000 in total cellular RNA). If the nu-

0036-8075/79/0420-0314$00.50/0

clear RNA were heavily contaminated by cytoplasmic RNA, we would expect the relative concentration of these two mRNA's to be similar in the nuclei and cytoplasm. Taken together, these observations suggest that the $mRNA_{ov}$ sequences observed in nuclear RNA are not due to contamination by $mRNA_{ov}$ in cytoplasm.

In previous experiments (*12*), hybridization of the 2.4- and 1.8-kb probes to nuclear RNA, fractionated by agarose gel electrophoresis under denaturing conditions, revealed that most of the intervening sequences, if not all, are hybridized together with structural gene sequences to discrete RNA bands that are larger than the mature $mRNA_{ov}$ (*12*). In addition, the apparent rate of RNA synthesis in vitro from structural and intervening sequences in isolated nuclei is very similar (*12*). These results suggest that structural sequences and intervening sequences are transcribed as one unit. Recently, Tilghman *et al.* (*16*) also demonstrated that 15*S* globin mRNA precursor contains both structural and intervening sequences. This indicates that all of the globin gene sequences, structural and intervening, are transcribed as one unit. Therefore, the difference in cellular abundance of the transcripts from structural and intervening sequences appears to result not from differing synthetic rates but from differential rates of degradation. That there is a substantial difference in the ratio of $mRNA_{ov}$ to intervening sequence RNA in the nucleus (tenfold) and cytoplasm (2000-fold) suggests that either the intervening sequences are rapidly processed (degraded) as soon as they reach the cytoplasm or, more likely, that they are degraded in the nucleus. Our detection of multiple bands of putative ovalbumin mRNA precursor or processing intermediates in the nucleus seems to favor the latter possibility. The small amount of intervening sequence RNA detected in polysomes may, in fact, be due to contamination of the polysome preparation by nuclear leakage.

There is a maximum of 20 copies of RNA transcripts from intervening sequences associated with polysomes even if we assume that no contamination from nuclear leakage occurred. This value is substantially lower than the abundant number of cytoplasmic mRNA sequences derived from other hormone-dependent structural gene sequences, such as ovalbumin, ovomucoid, conalbumin, and lysozyme (*15, 17, 18*) and suggests that RNA transcripts from intervening sequences within the ovalbumin gene do not function as components of mRNA for these other hormone-dependent genes. Furthermore, the ovalbumin gene intervening sequences cannot be detected in other tissues such as liver and spleen (less than 0.1 copy per cell, data not shown). Thus, it appears unlikely that the ovalbumin intervening sequences ever serve as templates for synthesis of any

Table 1. Ovalbumin (OV) RNA in various RNA preparations.

Sequence of OV gene transcripts	$C_0t_{1/2}$	Fraction of OV RNA sequence in total RNA*	Ratio of RNA to DNA†	Number of molecules per tubular gland cell‡
		Total RNA		
$mRNA_{ov}$	5.4×10^{-1}	5.6×10^{-3}	3.5	58,000
RNA 2.4	1.4×10^{2}	2.1×10^{-5}	3.5	228
RNA 1.8	1.4×10^{2}	1.8×10^{-5}	3.5	233
		Nuclear RNA		
$mRNA_{ov}$	9.2×10^{-1}	3.3×10^{-3}	0.25	2,500
RNA 2.4	1.0×10^{1}	3.0×10^{-4}	0.25	233
RNA 1.8	1.0×10^{1}	2.7×10^{-4}	0.25	250
		Polysomal RNA		
$mRNA_{ov}$	5.5×10^{-1}	5.5×10^{-3}	2.9	47,000
RNA 2.4	1.3×10^{3}	2.3×10^{-6}	2.9	20
RNA 1.8	1.3×10^{3}	2.0×10^{-6}	2.9	21

*Estimated by comparing the $C_0t_{1/2}$ of individual RNA with $C_0t_{1/2}$ of back hybridization between mRNA and its DNA probe. The $C_0t_{1/2}$ of back hybridization for $mRNA_{ov}$ is 3×10^{-3}. The $C_0t_{1/2}$ for back hybridization of intervening sequences in RNA 1.8 and RNA 2.4 to their probes are calculated to be 2.65×10^{-3} and 2.98×10^{-3}, respectively, after their size has been taken into account (*12*). The $C_0t_{1/2}$ values were obtained from the second phase of the hybridization curve. Therefore, the value represents only the hybridization of intervening sequences. †The RNA/DNA ratio in oviduct cells was obtained as previously described (*4*). ‡Calculated as described (*12*) after it was taken into account that only 80 percent of the oviduct cells are tubular gland cells and the $mRNA_{ov}$ sequences are 1930 nucleotides in size. Intervening sequences in OV1.8 are 1500 nucleotides and intervening sequences in OV2.4 are 1900 nucleotides.

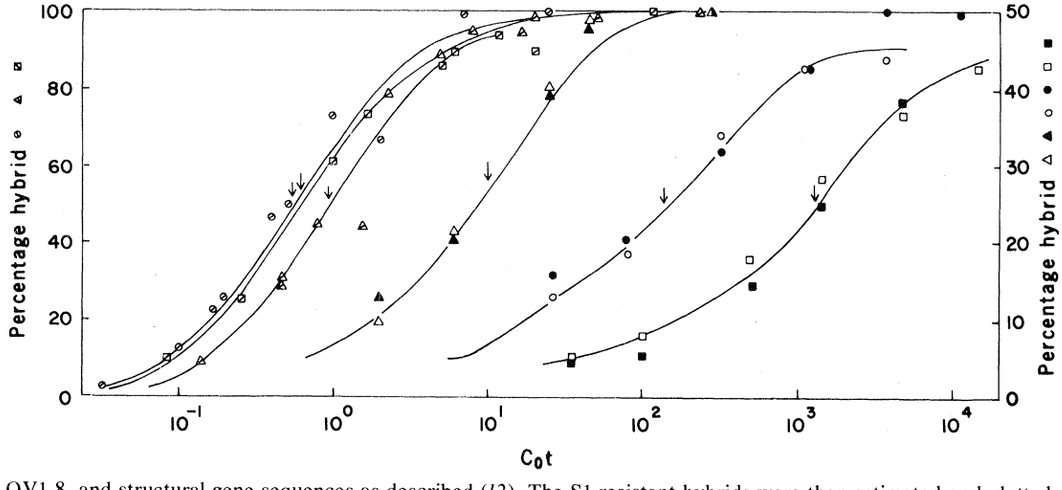

Fig. 1. Hybridization of mRNA's to various DNA probes. Nuclei were isolated from oviducts of chicks that received 20 mg of diethylstilbestrol pellet injection weekly for 2 weeks as described (*12*). Polysomes were isolated from chick oviducts according to the procedure of Palacios *et al.* (*14*). RNA's were then isolated from whole oviduct tissue, nuclei, or polysomes according to the procedure of Roop *et al.* (*12*). Isolated RNA's were then hybridized to nick-translated probes of OV2.4, OV1.8, and structural gene sequences as described (*12*). The S1 resistant hybrids were then estimated and plotted against the C_0t value. The RNA's used were total oviduct cellular RNA—⊘, ●, ○; nuclear RNA—◬, ▲, △; and polysomal RNA—◪, ■, □. The probes used were mRNA probe isolated from p0V230 DNA—⊘, ◬, ◪; 0V2.4 probes—●, ▲, ■; and 0V1.8 probes—○, △, □. For hybridization to structural probe (0.016 μg) the amounts of RNA used were 0.45 μg for nuclear RNA, 0.4 to 2 μg for total cellular RNA, and 1 μg for polysomal RNA. For hybridization to OV1.8 (0.015 ng) and OV2.4 (0.01 ng) probes, the amounts of RNA used were 25 μg for nuclear RNA, 400 μg for total cellular RNA, and 400 μg for polysomal RNA.

other mRNA sequences in these tissues.

In conclusion, we have used pure molecular hybridization probes for ovalbumin mRNA and intervening sequence RNA to measure their concentration in the nucleus and cytoplasm of the hormone-stimulated oviduct cell. We found that, unlike mRNA transcripts of the structural sequences of the avian ovalbumin gene, nuclear intervening sequence RNA was preferentially turned over and not transported efficiently to the polysomes.

MING-JER TSAI
SOPHIA Y. TSAI
BERT W. O'MALLEY
Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

References and Notes

1. Intervening sequences in eukaryotic DNA interrupt the structural DNA sequences coding for mRNA. Intervening sequences were first observed by Leder, Tonegawa, and other workers [see (*8, 9*)] in globin and immunoglobulin systems and were subsequently observed in several other systems (*8–11*).
2. B. W. O'Malley and A. R. Means, *Science* **183**, 610 (1974); B. W. O'Malley, W. L. McGuire, P.O. Kohler, S. G. Korenman, *Recent Prog. Horm. Res.* **25**, 105 (1969); W. T. Schrader and B. W. O'Malley, in *Receptors and Hormone Action*, B. W. O'Malley and L. Birnbaumer, Eds. (Academic Press, New York, 1977), vol. 2, pp. 189–224.
3. R. F. Cox, M. E. Haines, S. Emtage, *Eur. J. Biochem.* **49**, 225 (1975); G. S. McKnight, P. Pennequin, R. T. Schimke, *J. Biol. Chem.* **250**, 8105 (1975).
4. S. E. Harris, J. M. Rosen, A. R. Means, B. W. O'Malley, *Biochemistry* **14**, 2072 (1975).
5. R. D. Palmiter, P. B. Moore, E. R. Mulvihill, *Cell* **8**, 557 (1975).
6. E. C. Lai, S. L. C. Woo, A. Dugaiczyk, J. F. Catterall, B. W. O'Malley, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2205 (1978).
7. R. Breathnach, J. L. Mandel, P. Chambon, *Nature (London)* **270**, 314 (1977); M. T. Doel, M. Houghton, E. A. Cook, N. H. Carey, *Nucleic Acids Res.* **4**, 3701 (1977); R. Weinstock, R. Sweet, M. Weiss, H. Cedar, R. Axel, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1299 (1978).
8. A. J. Jeffreys and R. A. Flavell, *Cell* **12**, 1097 (1977); S. M. Tilghman, D. C. Tiemeier, F. Polsky, M. H. Edgell, J. G. Seideman, A. Leder, L. L. Enquist, B. Norman, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4406 (1977).
9. C. Brack and S. Tonegawa, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5652 (1977); P. Valenzuela, A. Venegas, F. Weinberg, R. Bishop, W. J. Rutter; *ibid.* **75**, 190 (1978); H. M. Goodman, M. V. Olson, B. D. Hall, *ibid.* **74**, 5453 (1977).
10. A Dugaiczyk, S. L. C. Woo, E. C. Lai, M. L. Mace, L. McReynolds, B. W. O'Malley, *Nature (London)* **274**, 328 (1978).
11. S. L. C. Woo, A. Dugaiczyk, M.-J. Tsai, E. C. Lai, J. F. Catterall, B. W. O'Malley, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3688 (1978).
12. D. R. Roop, J. L. Nordstrom, S. Y. Tsai, M.-J. Tsai, B. W. O'Malley, *Cell* **15**, 671 (1978).
13. L. A. McReynolds, J. F. Catterall, B. W. O'Malley, *Gene* **2**, 217 (1977).
14. R. Palacios, R. D. Palmiter, R. T. Schimke, *J. Biol. Chem.* **247**, 2316 (1972).
15. S. Y. Tsai, D. R. Roop, M.-J. Tsai, J. P. Stein, A. R. Means, B. W. O'Malley, *Biochemistry* **17**, 5773 (1978).
16. S. M. Tilghman, P. J. Curtis, D. C. Tiemeier, P. Leder, C. Weissmann, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1309 (1978).
17. N. E. Hynes, B. Groner, A. E. Sippel, M.C. Nguyen-Huu, G. Schütz, *Cell* **11**, 923 (1977).
18. D. C. Lee, G. S. McKnight, R. D. Palmiter, *J. Biol. Chem.* **253**, 3494 (1978).
19. We thank M. Vinion and C. Engleking for technical assistance and D. Roop, J. Nordstrom, and G. Lawson for helpful discussion. This work was supported by NIH grants HD-7857 and HD-8188.

19 September 1978

Chloroxymorphamine, an Opioid Receptor Site–Directed Alkylating Agent Having Narcotic Agonist Activity

Abstract. *Chloroxymorphamine, the 6β-N,N-bis(2-chloroethyl) derivative of oxymorphone, is a potent nonequilibrium narcotic agonist in the longitudinal muscle preparation of guinea pig ileum. The corresponding naltrexone analog, chlornaltrexamine, is a potent nonequilibrium antagonist of morphine. These receptor site–directed alkylating agents possess considerable potential as pharmacologic and biochemical probes of opioid receptors.*

The development of highly selective opioid receptor site–directed alkylating agents that act both in vivo and in vitro should contribute significantly to the armamentarium of compounds that are employed as investigational tools. We reported (*1*) on an alkylating analog of naltrexone, chlornaltrexamine (CNA) (Fig. 1), which affords long-lasting narcotic antagonism in mice and exhibits avid stereospecific binding in vitro. We now report on a second compound, chloroxymorphamine (COA) (Fig. 1), whose interaction with opioid receptors leads to sustained agonism. We also report on experiments that further demonstrate the nonequilibrium nature and specificity of CNA.

Since CNA is a potent antagonist, we postulated that an identical modification of oxymorphone (Fig. 1) (*2*), a potent analgesic, might afford an affinity labeling agent, COA, with agonist properties. The synthesis of COA, which is similar to that for CNA (*1*), involves $NaCNBH_3$, reduction of oxymorphone in the presence of diethanolamine, followed by treatment of the bis(hydroxyethyl)amino intermediate with CCl_4-Ph_3P. After washing with hot ethyl acetate, the crude COA was purified on a dry silica-gel column and eluted with ether-methanol-am-

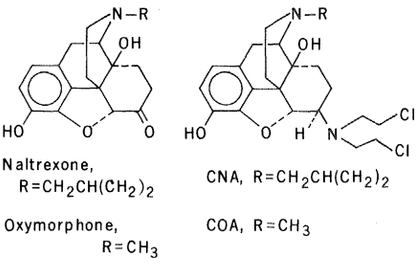

Fig. 1. The structures of oxymorphone, chloroxymorphamine (COA), naltrexone, and chlornaltrexamine (CNA).

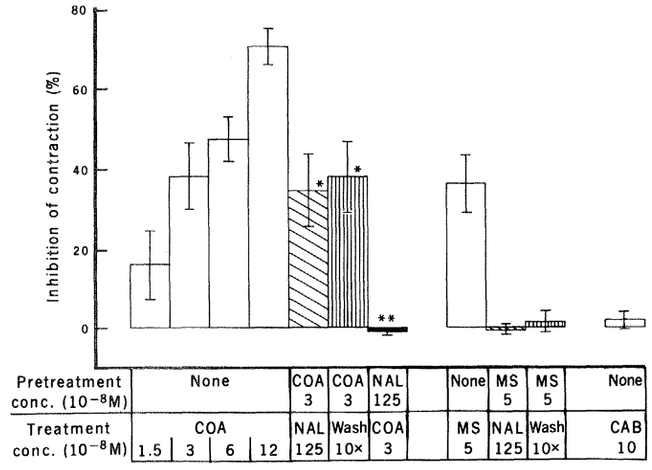

Fig. 2. The effect of chloroxymorphamine on the coaxially stimulated longitudinal muscle of guinea pig ileum. The muscle was prepared by the method of Rang (*4*). A strip of longitudinal muscle was removed with myenteric plexus intact from a segment of the guinea pig ileum. A 1- to 2-cm portion of this muscle was suspended between two platinum electrodes in a 10-ml bath containing Krebs-Ringer bicarbonate solution with 1.25 μM chlorpheniramine maleate. The organ bath was bubbled with a mixture of 95 percent O_2 and 5 percent CO_2 while being maintained at 36° to 37°C. Contractions in the muscle strip were initiated by supramaximal rectangular pulses (80 V, 0.5-msec duration, 0.1 Hz) and recorded by an isometric transducer connected to one end of the tissue. After equilibration the ileal muscles were exposed to either chlorambucil (*CAB*), morphine sulfate (*MS*), or chloroxymorphamine (*COA*) for 15 minutes or naloxone (*NAL*) for 5 minutes. Open bars represent percentage of inhibition of muscle twitch after exposure to these drugs. A computer program was used to analyze the results (*8*). Diagonally hatched bars represent percentage of inhibition remaining after subsequent treatment with naloxone. Vertically hatched bars represent percentage of inhibition remaining after the treated preparations were washed ten times with Krebs-Ringer bicarbonate solution. The solid bar represents percentage of inhibition observed 15 minutes after COA treatment of muscle strips that had been treated with naloxone. Each value given is the mean ± standard error of three to seven experiments; *, significantly different ($P < .05$) from similarly treated morphine control; **, significantly different ($P < .05$) from nonpretreated control.

0036-8075/79/0420-0316$00.50/0