RNA), in cytoplasmic mRNA of HeLa cells (*2, 3*), and in mouse ascites cells (*4*). We now show that similar poly(A) sequences are found in rapidly labeled RNA of chick polysomes, presumed to be mRNA. The function of these sequences is unknown, but it seems likely that they function in the synthesis of mRNA or its transport to the cytoplasm or in its translation or a combination of these processes. A direct function of poly(A) in the translation process is suggested by the finding that adenine-rich sequences are present in mRNA synthesized by viral RNA polymerases in vaccinia (*5*) and vesicular stomatitis (*6, 16*) virions, both of which reproduce in the host cell cytoplasm. Since the vaccinia mRNA is transcribed from a double-stranded DNA genome and the vesicular stomatitis mRNA from a single-stranded RNA genome, the common occurrence of poly(A) in these mRNA's seems more likely to be related to their role in translation than to their synthesis. Poly(A) has also been observed in the mRNA of adenovirus (*7*), which is synthesized in the host cell nucleus but transported to the cytoplasm for translation (*17*).

Our demonstration of poly(A) sequences in virion RNA from poliovirus and EEE virus provides additional evidence consistent with a functional role of poly(A) in the process of translation of mRNA in animal cells. Both of these viral RNA's are known (*18*) to be infectious when purified from their respective virions, so that they are capable of functioning as mRNA's with their host cell translation mechanism. The common presence of poly(A) sequences is especially significant because of the dissimilarity of these viruses. For example, EEE, like other group A arboviruses, has a lipoprotein envelope about its capsid, while poliovirus, of the picornaviruses, has none. The base compositions of the virion RNA's of these viruses are also quite different [data above and (*13*)]. The significant chemical feature which EEE virus and poliovirus do have in common is a poly(A) sequence in their virion RNA; hence it seems reasonable to hypothesize that these sequences play some common role in the functioning of the RNA. While several possibilities might be raised, the one process in which poliovirus RNA, EEE virus RNA, vaccinia virus mRNA, vesicular stomatitis virus mRNA, and the host cell mRNA's all function in common is clearly translation. We have no direct evidence at present as to what function poly(A) might play in the translation process.

The presence of small poly(A) sequences that can be accurately analyzed in viral RNA molecules is also significant from another standpoint, the physical-chemical characterization of that RNA. As an example, the analytical data regarding poliovirus RNA poly-(A) in Table 1, together with the known base composition of poliovirus RNA (*13*) permit a direct calculation of the molecular weight of the polio RNA: 2.77×10^6. This is in excellent agreement with the figure previously determined by physical methods: 2.4 to 2.5×10^6 (*12*). This calculation is possible only when the size of the poly-(A) sequence is accurately known, and therefore we are unable at present to apply it to EEE virion RNA.

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References and Notes

1. L. Lim and E. S. Canellakis, *Nature* **227**, 710 (1970).
2. M. Edmonds, M. H. Vaughan, H. Nakazato, *Proc. Nat. Acad. Sci. U.S.* **68**, 1337 (1971).
3. J. E. Darnell, R. Wall, R. J. Tushinski, *ibid.*, p. 1321.
4. S. Y. Lee, J. Mendecki, G. Brawerman, *ibid.*, p. 1331.
5. J. Kates, *Cold Spring Harbor Symp. Quant. Biol.* **35**, 743 (1970); J. Kates and J. Beeson, *J. Mol. Biol.* **50**, 19 (1970).
6. J. A. Mudd and D. F. Summers, *Virology* **42**, 958 (1970).
7. L. Philipson, R. Wall, G. Glickman, J. E. Darnell, *Proc. Nat. Acad. Sci. U.S.* **68**, 2806 (1971).
8. E. R. Pfefferkorn and H. S. Hunter, *Virology*, **20**, 433 (1963).
9. B. A. Phillips, D. F. Summers, J. V. Maizel, *ibid.* **35**, 216 (1968)
10. J. R. Warner, R. Soeiro, H. C. Birnboim, J. E. Darnell, *J. Mol. Biol.* **19**, 349 (1966).
11. J. H. Strauss, R. B. Kelly, R. L. Sinsheimer, *Biopolymers* **6**, 793 (1968).
12. N. Granboulan and M. Girard, *Virology* **4**, 475 (1969); G. A. Tannock, A. J. Gibbs, P. D. Cooper, *Biochem. Biophys. Res. Commun.* **38**, 298 (1970).
13. The nucleotide composition of poliovirus virion RNA was found by P. Roy and J. Bishop [*Virology* **6**, 604 (1970)] to be 30.3 percent AMP, 23.3 percent UMP, 22.4 percent GMP, and 24.1 percent CMP.
14. J. A. Armstrong, unpublished observations.
15. ———, L. C. Freeburg, M. Ho, *Proc. Soc. Exp. Biol. Med.* **137**, 13 (1971).
16. D. Baltimore, A. S. Huang, M. Stampfer, *Proc. Nat. Acad. Sci. U.S.* **66**, 572 (1970).
17. J. T. Parsons, J. Gardner, M. Green, *ibid.* **68**, 557 (1971); H. J. Raskas and C. K. Okubo, *J. Cell Biol.* **49**, 438 (1971).
18. E. Wecker, *Virology* **7**, 241 (1959); J. J. Holland, L. C. McLarne, J. T. Syverton, *J. Exp. Med.* **110**, 65 (1959).
19. J. R. Coleman and A. W. Coleman, *J. Cell Physiol.* **72**, Suppl. 1, 19 (1968).
20. R. K. Morse, H. Herrmann, S. M. Heywood, *Biochim. Biophys. Acta* **232**, 403 (1971).
21. We thank Dr. Richard Morse for aid in preparing labeled polysomal RNA from chicks. This work was supported by grants AM-10074, CA-10922, AI-02953, and AI-08368 from the National Institutes of Health and by a career development award of the U.S. Public Health Service to M.H.V.

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Receptor-Estrogen Complex in the Nuclear Fraction of Rat Uterine Cells during the Estrous Cycle

Abstract. *A quantitative method was used to determine the concentration of receptor-estrogen complex in the nuclear fraction of rat uterine cells throughout the estrous cycle. The concentrations of nuclear receptor-estrogen complex were: metestrus, 0.22; diestrus, 0.75; proestrus, 1.29; and estrus, 0.31 picomoles per milligram of DNA. This cyclic fluctuation in the nuclear complex closely parallels the secretion of ovarian estrogen during the estrous cycle, an indication that the accumulation of receptor-estrogen complex by the nuclear fraction of uterine cells may be of physiological significance, and under the control of endogenous estrogen.*

Estrogenic hormones are selectively retained by the uterus and other tissues sensitive to estrogen (*1*). It is generally accepted that the interaction of estrogen (E) with the uterus is characterized by the binding of E to a cytoplasmic protein (R) with the subsequent movement of the receptor-estrogen complex (RE) from the cytoplasm to the nucleus of uterine cells. The appearance of the RE complex in the nuclear fraction of the uterine cells is a specific process initiated by estrogenic hormones, but not by progesterone or testosterone (*1–3*). It has been suggested that the nuclear RE complex

mediates the primary action of the hormone (*4*). Previous reports of the translocation and accumulation of the RE complex in the nucleus have been based on studies of tissues that had been exposed to exogenous estrogen. It has been assumed that the observed translocation and accumulation of the RE complex under these conditions also occurs in vivo in response to circulating endogenous estrogen. If these processes are of physiological significance they should occur during the estrous cycle in response to changing concentrations of estrogens in blood. We have examined the concentration of nuclear RE in rat uterine cells throughout the estrous cycle with a new assay based on the exchange of [^{3}H]estradiol with estradiol bound in the nuclear fraction. We undertook these studies to define the relation between the concentration of nuclear RE complex and the hormonal state of the animal.

Virgin female rats (7 to 9 months old, 250 to 350 g) of the Purdue-Wistar strain were housed in a controlled environment (21.1° to 22.2°C humidity 45 to 55 percent) with 13 hours of artificial light between 7 a.m. and 8 p.m. Vaginal smears were made every morning, and rats that showed a 4-day cycle were studied after the completion of at least three normal cycles. The animals were killed by decapitation at noon on the days of proestrus, estrus, metestrus, or diestrus. The uteri were cleaned of adhering fat and mesentery, slit longitudinally to remove excess intraluminal fluid, and weighed. One horn of each uterus was examined for total protein and DNA content (*5*). The remaining horn was analyzed for specific nuclear binding sites by the nuclear exchange method (*3, 6*).

The nuclear exchange assay consisted of the incubation of washed nuclear fractions from uterine cells at 37°C for 30 minutes in the presence of [^{3}H]estradiol alone, series A [(0.8, 1.7, 3.3, and 5) $\times 10^{-9}M$)] or [^{3}H]-estradiol [(0.8, 1.7, 3.3, and 5) $\times 10^{-9}M$)] plus diethystilbestrol (DES) at 100 times the concentration of [^{3}H]-estradiol, series B. After incubation, the fractions were washed, and the [^{3}H]estradiol was extracted and measured in a scintillation counter. The quantity of specifically bound [^{3}H]estradiol was determined by subtraction of nonspecifically bound [^{3}H]estradiol, series B ([^{3}H]estradiol bound in the presence of a hundredfold excess of DES) from total [^{3}H]estradiol binding, series A. The concentration of specific nuclear RE was determined by a double reciprocal plot of the specifically bound [^{3}H]estradiol.

Table 1. Changes in the quantity of RE complex in the uterine nuclear fraction during the estrous cycle. Results are expressed as means ± standard error of the mean (five to six determinations).

Stage	Uterus (pmoles)	Nuclear RE, concentration in:		$K^R_d \times 10^9$ (M)
		Protein (pmole/100 mg)	DNA (pmole/mg)	
Metestrus	0.90 ± 0.25	0.86 ± 0.26	0.22 ± 0.06	2.6 ± 0.8
Diestrus	3.16 ± 0.72	2.69 ± 0.67	0.75 ± 0.20	1.6 ± 0.4
Proestrus	4.53 ± 0.59	3.36 ± 0.39	1.29 ± 0.24	1.4 ± 0.3
Estrus	0.96 ± 0.23	0.82 ± 0.23	0.31 ± 0.09	1.8 ± 0.4

The concentration of specific estrogen binding sites, that is, RE complex, in the uterine nuclear fraction throughout the rat estrous cycle is shown in Table 1. The number of binding sites per milligram of DNA is at a minimum in estrus and metestrus (approximately 1000 sites per nucleus). The concentration increases sharply between metestrus and diestrus (approximately 3500 sites per nucleus), $P < .05$, and reaches a maximum on the day of proestrus (approximately 5000 sites per nucleus), $P < .05$ (*7*). This relation between the amount of RE per nucleus and the stage of the estrous cycle is also evident when nuclear RE is compared with the weight or protein content of the uterus (Table 1).

Uterine weight, protein content, and the ratio of protein to DNA (not shown) are all significantly higher in proestrus than in metestrus or diestrus ($P < .05$), suggesting that fluctuations in protein synthetic activity of the uterus occur during the estrous cycle (Table 2). Similar observations have been reported for these and other uterine responses throughout the estrous cycle (*8*). In addition, it has been demonstrated that these responses are initiated by estrogenic hormones (*9*). Thus, maximum estrogenic responses are accompanied by peak concentrations of nuclear RE complex in proestrus uterine cells.

The cyclic fluctuation in the concentration of nuclear RE from uterine cells during the estrous cycle closely parallels the rate of ovarian estrogen secretion (*10*). The low concentration of nuclear RE during estrus and metestrus is accompanied by a minimum rate of ovarian estrogen secretion. During diestrus the rate of estrogen secretion increases (*10*), and this increase is accompanied by an elevation of the content of nuclear RE over that present during estrus and metestrus (our results); in addition, the maximum concentration of nuclear RE during proestrus (Table 1) coincides with the peak of estrogen secretion (*10*). This correlation suggests that the cyclic fluctuation of uterine RE content is under the control of ovarian estrogens.

One could argue that the variation in nuclear RE content during the estrous cycle is a result of periodic alterations in uterine cytoplasmic components, for example, cytoplasmic receptor molecules, that are necessary for the appearance of RE in the nuclear fraction. In addition, the increase in cytoplasmic components (see Table 2) could result in artifactual variations in "nuclear RE" due to contamination of the nuclear preparation with cytoplasmic proteins. To study these possibilities, we injected rats in proestrus and metestrus subcutaneously with 10 μg of estradiol, and subjected the uteri to the nuclear exchange assay after 1 hour. Estradiol treatment either in proestrus, when total receptor might be high, or in metestrus, when total receptor might be low, results in equivalent concentrations of nuclear RE (metestrus, 8.38 ± 1.04 pmole/g; proestrus, 8.31 ± 1.42 pmole/g).

The cyclic fluctuation in nuclear RE during the estrous cycle reflects the

Table 2. Changes in uterus during the estrous cycle. Results are expressed as means ± standard error of the mean (eight to twelve determinations).

Stage	Weight (mg)	Uterine content of:	
		Protein (mg)	DNA (mg)
Metestrus	674 ± 28	102 ± 7	3.64 ± .22
Diestrus	747 ± 73	103 ± 12	3.59 ± .42
Proestrus	973 ± 81	129 ± 3	3.63 ± .24
Estrus	775 ± 60	116 ± 8	3.34 ± .22

change in the distribution of estrogen receptors between the cytoplasm and nucleus. Estrogen treatment is known to promote a depletion of cytoplasmic receptors that is accompanied by a parallel increase in nuclear RE (*11*). Furthermore, Lee and Jacobson (*12*) have shown that the concentration of free cytoplasmic receptors is at a minimum in proestrus, and at a maximum in estrus. Thus, the ratio of nuclear RE (our results) to free cytoplasmic binding sites (*12*) is high when the rate of estrogen secretion is at a maximum in proestrus (*10*). Conversely, the ratio of nuclear RE (our results) to free cytoplasmic sites (*12*) is low when the rate of estrogen secretion is at a minimum, as in estrus (*10*).

The linearity of the double reciprocal plots, and the similarity in the K^R_d (*13*) (1.2×10^{-9} to $2.6 \times 10^{-9} M$, 37°C) throughout the estrous cycle (Table 1), and after estradiol treatment, suggest that the receptors are homogenous in their affinity for estradiol. These K^R_d values are in agreement with those obtained by others for both cytoplasmic and nuclear estrogen binding sites (*3, 14*).

A causal relation between the elevated uterine weight, the protein content or the protein to DNA ratio, and the maximum concentration of nuclear RE during proestrus remains to be established. Estrogen is known to augment uterine protein and RNA synthesis, and these effects are blocked by puromycin and actinomycin (*15*). Moreover, the RE complex has been implicated in the estrogen stimulation of RNA polymerase activity (*16*). While the possibility that the estrogen receptor exerts its effects at the cytoplasmic level cannot be excluded, the results of our study suggest that nuclear RE complexes may be of physiological importance, and are not simply a pharmacologic phenomenon.

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References and Notes

1. J. Gorski, D. Toft, G. Shyamala, D. Smith, A. Notides, *Recent Progr. Horm. Res.* **24**, 45 (1968); E. V. Jensen, M. Numata, S. Smith, T. Suzuki, E. R. DeSombre, *Develop. Biol. Suppl.* **3**, 151 (1969).
2. G. A. Puca and F. Bresciani, *Nature* **218**, 967 (1968).
3. J. Anderson, J. H. Clark, E. J. Peck, Jr., *Biochem. J.* **126**, 561 (1972).
4. E. V. Jensen, T. Suzuki, M. Numata, S. Smith, E. R. DeSombre, *Steroids* **13**, 417 (1969).
5. Protein was assayed by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951). DNA was assayed by the method of G. Ceriotti, *J. Biol. Chem.* **198**, 297 (1952).
6. The nuclear exchange method is based on the observation that estrogen, previously complexed with receptor and bound to the cell nucleus as RE as a result of hormonal injections or ovarian secretion, is freely exchangeable with [^{3}H]estradiol during in vitro incubations of the nuclear fraction. The amount of [^{3}H]estradiol specifically exchanged during the incubation period is proportional to the amount of RE in the nuclear fraction.
7. Statistical differences were determined by an analysis of variance, followed by the method of least significant differences.
8. E. B. Astwood, *Amer. J. Physiol.* **126**, 162 (1939); I. Noack and H. Schmidt, *Endokrinologie* **53**, 291 (1968); Y. N. Sinha and H. A. Tucker, *Proc. Soc. Exp. Biol. Med.* **131**, 908 (1969); C. Desjardins, V. M. Chapman, F. H. Bronson, *Anat. Rec.* **167**, 465 (1970).
9. S. Brody and N. Wiquist, *Endocrinology* **68**, 971 (1961); H. Schmidt, I. Noack, H. Walther, K. D. Voigt, *Acta Endocrinol.* **56**, 231 (1967); B. Shirley, J. Wolinsky, N. B. Schwartz, *Endocrinology* **82**, 959 (1968).
10. T. Hori, M. Ide, T. Miyake, *Endocrinol. Jap.* **15**, 215 (1968); K. Yoshinaga, R. A. Hawkins, J. F. Stocker, *Endocrinology* **85**, 175 (1969).
11. G. Giannopoulos and J. Gorski, *J. Biol. Chem.* **246**, 2524 (1971).
12. C. Lee and H. I. Jacobson, *Endocrinology* **88**, 596 (1971).
13. K^R_d, the dissociation constant of the complex of receptor and estrogen.
14. D. Toft and J. Gorski, *Proc. Nat. Acad. Sci. U.S.* **55**, 1574 (19(D. Toft, G. Shyamala, J. Gorski, *Proc. Nat. Acad. Sci. U.S.* **57**, 1740 (1967); J. H. Clark and J. Gorski, *Biochim. Biophys. Acta* **192**, 508 (1969); T. Erdos, R. Bessada, J. Fries, *Fed. Eur. Biochem. Soc. Lett.* **5**, 161 (1969); G. Puca and F. Bresciana, *Nature* **223**, 745 (1969); G. Shyamala and J. Gorski. *J. Biol. Chem.* **244**, 1097 (1969); E. E. Baulieu and J. P. Raynaud, *Europ. J. Biochem.* **13**, 293 (1970).
15. W. D. Noteboom and J. Gorski, *Proc. Nat. Acad. Sci. U.S.* **50**, 250 (19 H. Ui and G. C. Mueller, *ibid.*, p. 256; J. Gorski, *J. Biol. Chem.* **239**, 889 (1964); T. H. Hamilton, C. C. Widnell, J. R. Tata, *Biochim. Biophys. Acta* **108**, 165 (1965).
16. C. Raynaud-Jammet and E. E. Baulieu, *C. R. Acad. Sci. Sér. D* **268**, 3211 (1969); D. Hough, M. Arnaud, M. Mousseron-Canet, *ibid.* **271**, 603 (1970).
17. This work was supported by grants from the National Institutes of Health (HD 04985), and from the Research Corporation, Atlanta, Georgia. We thank T. Cetti and J. Haselby for technical assistance.

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Synaptogenesis in the Rat Cerebellum: Effects of Early Hypo- and Hyperthyroidism

Abstract. *The number of synapses in the molecular layer of the rat cerebellum is reduced by early hypo- and hyperthyroidism within 30 days. Hypothyroidism retards synaptogenesis after 10 days, while hyperthyroidism accelerates synaptogenesis initially, but by 21 days the number of synapses is reduced. The sensitivity of developing synapses to thyroid hormone may permit analysis of the events triggering synaptogenesis.*

Neonatal hypo- and hyperthyroidism produce various deficits in postnatal neural development, including a decrease in cerebral and cerebellar weights (*1, 2*), and changes in the number, size, and packing density of cells (*1–3*). Changes in the amount and composition of the neuropil have been demonstrated in the sensorimotor cortex (*2, 4*), the visual cortex (*5*), and the cerebellum (*6*). Changes have also been found in development of metabolic compartmentation, which is thought to reflect maturation of dendritic processes and nerve terminals (*7*). Together these results indicate a retardation of neuropil development in hypothyroidism, and an acceleration in hyperthyroidism. Also, there is behavioral and electrophysiological evidence for neurological changes in these conditions in the form of retarded or accelerated maturation of innate behavioral patterns (*8, 9*), and abnormalities in the electrical patterns of the brain (*8, 10*).

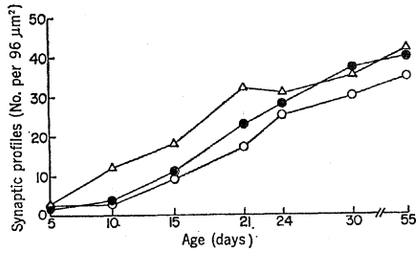

Fig. 1. Density of synaptic profiles in cerebellar molecular layer. △, Hyperthyroid; ○, hypothyroid; ●, control. Statistical significance: control and hypothyroid, at 21 and 30 days, $P < .01$; at 55 days, $P < .05$; control and hyperthyroid, at 10, 15, and 21 days, $P < .01$.

We examined the effects of early hypo- and hyperthyroidism on synaptogenesis in the cerebellar molecular layer with quantitative light and electron microscope methods. Our results provide evidence that both hypo- and hyperthyroidism cause a reduction in the total number of synapses formed in the cerebellar molecular layer, but by different processes.

Groups of animals were injected from birth with either physiological saline (controls), propylthiouracil (producing a hypothyroidism), or L-thy-