*PROSTATIC NUCLEAR CHROMATIN: AN EFFECT OF TESTOSTERONE ON THE SYNTHESIS OF RIBONUCLEIC ACID RICH IN CYTIDYLYL(3′,5′)GUANOSINE**

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Our studies[1, 2] revealed that RNA-synthesizing capacity at certain sections of prostatic chromatin is preferentially enhanced by injection of testosterone into castrated animals. Actinomycin D *in vivo* or *in vitro* can selectively suppress this enhancement of prostatic nuclear RNA synthesis by testosterone. RNA synthesized at the chromatin section where testosterone has a profound effect was found to be rich in guanine and cytosine. Experiments described in this paper show that whole prostatic nuclear chromatins can be assigned to four groups according to their ability and requirements in supporting the synthesis of different species of RNA by DNA-dependent RNA polymerases. We will show that the chromatin segment affected profoundly by testosterone *in vivo* is only a very small portion (1% or less) of total nuclear chromatin and that DNA associated with this particular section of chromatin is rich in deoxycytidylyl (3′,5′)deoxyguanosine.

Materials and Methods.—Disruption of purified nuclei[3, 4] was carried out in a French pressure cell (American Instrument Co.). Purified nuclei were suspended in 0.32 *M* sucrose–0.001 *M* $MgCl_2$ and the pressure gauge was maintained at 4000–6000 psi. Under a light microscope, it was seen that more than 90% of nuclei were ruptured by one passage of the nuclear suspension through the press cell. About 15% of the RNA polymerase activity associated with prostatic nuclei was lost after such a pressure treatment. The disrupted nuclei still retain the property of synthesizing GC-rich RNA which is selectively augmented by androgen. Actinomycin D was a generous gift of Merck, Sharp and Dohme Research Laboratories. *Micrococcus lysodeikticus* RNA polymerase was prepared by procedure A of Nakamoto, Fox, and Weiss[5] to step V. RNA synthesis by nuclei or purified bacterial polymerase was carried out in a final volume of 0.5 ml or 1 ml containing 0.1–0.3 mM of an αP^{32} nucleoside triphosphate (50–200 $\mu c/\mu mole$); 1 mM each of three other complementary nucleoside triphosphates; 1 mM $MnCl_2$; 5 mM $MgCl_2$; 20 mM β-mercaptoethanol; 60 mM KCl; 120 mM Tris-HCl buffer, pH 8.1. Unless otherwise stated, reactions were carried out for 15 min at 37°C. Other methods were described before.[1, 2] For the comparison of nearest-neighbor frequency (NNF) of different RNA, we found the following presentation very useful: (*a*) *NNF spectrum* refers to the plot shown in Figures 2 and 4 in which the ordinate is NNF and the abscissa, the dinucleotide sequences; (*b*) differential NNF spectrum, the extent of difference (in % changes) in NNF for each dinucleotide sequence at ordinate and the dinucleotide sequences along the abscissa (see Fig. 3). Several duplicate experiments showed that errors involved were usually below 5% of NNF and a difference in 10% or more was significant and was reproducible. Although the actual NNF values of RNA synthesized in the presence of prostatic nuclei may vary somewhat with different nuclear preparations from different groups of rats, the shapes of NNF spectra were remarkably similar (see Figs. 2 and 4 for NNF spectra of RNA synthesized by prostatic nuclei of testosterone-injected rats in two independent experiments).

Results.—RNA synthesis by prostatic nuclei in the presence and absence of micrococcus RNA polymerase: Various properties of RNA synthesis by prostatic nuclear systems and the effects of androgen *in vivo* on these systems have been reported elsewhere.[1, 2, 4, 6] When purified micrococcus RNA polymerase was added to these systems in the absence of exogenous DNA, RNA synthesis was enhanced considerably. With several preparations of prostatic nuclei, the extent of stimulation was

TABLE 1

RNA SYNTHESIS AND ACTINOMYCIN D BINDING BY RAT DNA AND PROSTATIC NUCLEAR PREPARATIONS*

DNA or Nuclei	Micrococcus polymerase	P^{32}-UMP incorporation ($\mu\mu$moles/100 μg DNA)	Per cent
Rat liver DNA	+	4300	100.0
Prostatic nuclei			
C	+	1080	25.1
T	+	1010	23.5
C	−	50	1.2
T	−	104	2.4
		Actinomycin-D binding (μg/100 μg DNA)	Per cent
Rat liver DNA	−	30–40	100
Prostatic nuclei (C or T)	−	7–10	20–30

* Prostatic nuclei were isolated from rats castrated for 60 hr and pressure-disrupted just before the addition. C: control castrates; T: castrated rats injected with testosterone at 0, 24, and 48 hr. Reactions were carried out in the presence of 2–6 μg rat liver DNA or prostatic nuclei which contain 10–30 μg DNA and an excess amount of micrococcus polymerase (10 μg) if added. They were incubated under the standard condition for 15 min. Under these conditions the amount of UMP incorporated was proportional to the amount of DNA used. Addition of twice the amount of micrococcus polymerase resulted in only about 20% increase in RNA synthesis in the tube which already had the bacterial polymerase. Binding of actinomycin D was analyzed spectrophotometrically under conditions where the amount of antibiotic bound was proportional to the amount of nuclei or DNA present and DNase pretreatment of nuclei or DNA abolished such binding.[7]

variable. This was apparently due to variations in the degree of integrity of nuclei since methods leading to the rupture of the nuclear membrane (exposure to hypotonic solution, freezing and thawing, or French pressure treatment) always increased the capacity of prostatic nuclei to support RNA synthesis (obviously by supplying DNA template) in the presence of additional bacterial polymerase. However, if nuclei were exposed to a controlled French pressure treatment, described in this paper, the extent of enhancement of RNA synthesis by bacterial polymerase was relatively constant. With many different preparations of prostatic

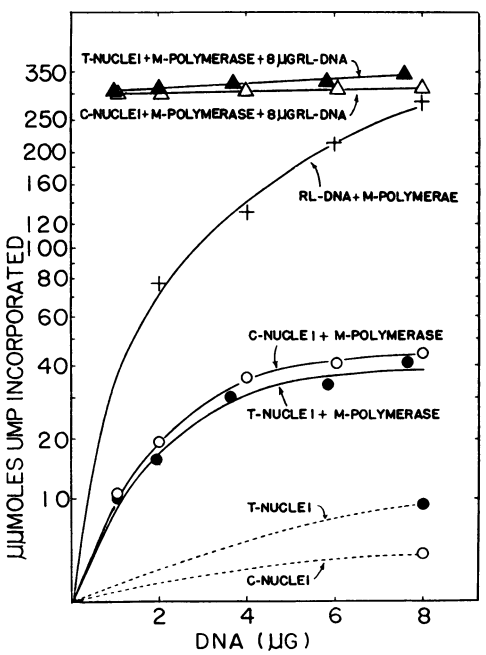

FIG. 1.—Relative template activity of rat liver DNA and pressure-disrupted prostatic nuclei. Incorporation of P^{32}-UMP was measured in the presence of micrococcus (M) polymerase (22 μg protein) using purified rat liver DNA (×) or prostatic nuclei (French pressed) from control (○) and testosterone-treated (●) castrated rats as the sources of DNA template. The amount of nuclei used is expressed as the amount of DNA associated. In some of the experimental tubes containing 8 μg of rat liver DNA, different amounts of prostatic nuclei from control (△) and testosterone-treated (▲) animals were added to examine the effect of nuclei on the template activity of rat liver DNA. For comparison, RNA polymerase activity of pressure-disrupted prostatic nuclei alone (– – – –) is also shown. Treatment of animals and other conditions are the same as described in Table 1.

nuclei, we found that (*a*) template activity (measured as the capacity to direct RNA synthesis by micrococcus polymerase) of pressure-disrupted prostatic nuclei is about 20–30 per cent that of purified rat liver DNA; (*b*) testosterone treatment of castrated rats for 10–70 hours enhanced the nuclear RNA polymerase activity of intact or pressure-disrupted prostatic nuclei but did not enhance the template activity of prostatic nuclei to support RNA synthesis when it was assayed with micrococcus polymerase; and (*c*) the amount of RNA synthesized by prostatic nuclei alone is about 5–10 per cent of that which can be synthesized by micrococcus polymerase in the presence of pressure-disrupted prostatic nuclei and is only 1–2 per cent of that which can be made by micrococcus polymerase using purified rat DNA as the template. Two representative experiments which led to these conclusions are shown in Table 1 and Figure 1. In Table 1, the amount of actinomycin D which can be *readily* bound by purified rat liver DNA or prostatic nuclei is shown. With many different preparations of intact or disrupted nuclei, the amount of actinomycin D *readily* bound by DNA associated with prostatic nuclei was about 20–30 per cent of that which could be bound by the same amount of purified rat DNA (since DNase abolished binding of actinomycin D by nuclear chromatin, the drug binding is apparently due solely to DNA associated with prostatic nuclei).[7] The good correlation between actinomycin binding capacity and template activity of prostatic nuclei and purified DNA suggested that in pressure-disrupted prostatic nuclei, a considerable portion of DNA which binds actinomycin can also act as a template for *in vitro* RNA synthesis by a bacterial polymerase. Figure 1 shows the apparent template activity of different concentrations of prostatic nuclei. The result is in agreement with that of Table 1. It is also shown that prostatic nuclei at the concentrations used did not significantly alter the template activity of purified DNA in micrococcus polymerase system.

NNF spectra of RNA synthesized at different regions of DNA: According to the current concept, the NNF of RNA synthesized by a DNA-dependent RNA polymerase should reflect the NNF of the DNA template utilized.[10] We have reported that RNA synthesized by prostatic nuclei of testosterone-injected animals is different in its NNF from that of control castrates[2] (Figs. 2 and 3). This fact suggested that DNA templates employed for the RNA synthesis in these two systems are not identi-

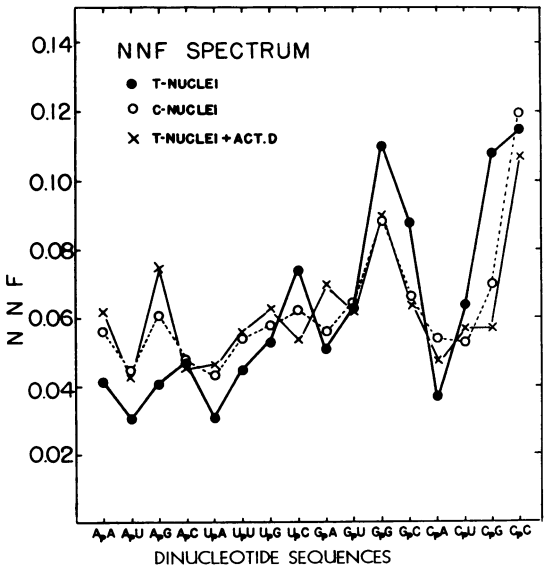

FIG. 2.—NNF spectrum of RNA synthesized *in vitro* by prostatic nuclei from control (○) and testosterone-treated (●) castrates. Rats were sacrificed 65 hr after castration. Testosterone injection was at 24, 48, and 63 hr after the surgery. If used (×), the amount of actinomycin D was 0.6 μg per ml of reaction mixture. The actual numbers for NNF have been reported in our preliminary communication.[2]

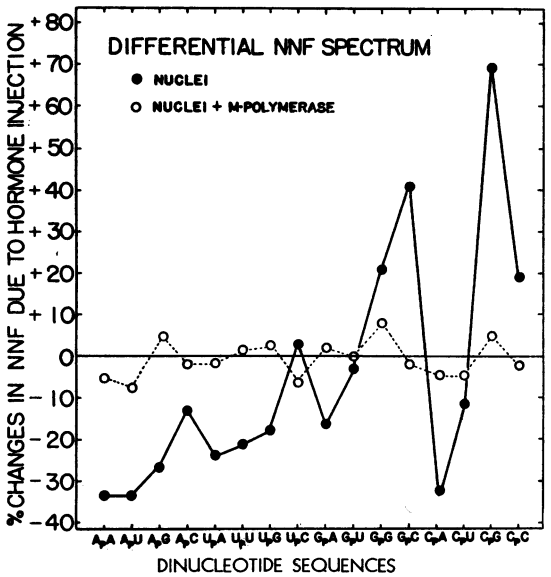

FIG. 3.—Changes in NNF of RNA synthesized by prostatic nuclei. Twenty rats were sacrificed after 60 hr and testosterone was injected to 10 rats at 34 and 12 hr before the sacrifice. RNA synthesized by prostatic nuclei alone (●) or by pressure-disrupted nuclei with micrococcus polymerase (○) was analyzed for NNF. Per cent change in NNF for each base pair was calculated from (NNF.T − NNF.C)/NNF.C × 100, where NNF.T and NNF.C were, respectively, NNF for a particular dinucleotide sequence in the RNA synthesized in the presence of prostatic nuclei from testosterone-treated and control castrates. Actual NNF values are presented in Figs. 2 and 4.

cal in their nucleotide sequences. On the other hand, the NNF spectra of RNA synthesized by prostatic nuclei without exogenous polymerase are clearly different from those of RNA synthesized by micrococcus polymerase using pressure-disrupted prostatic nuclei as the source of DNA template (Fig. 4). In addition, the NNF of RNA synthesized by the bacterial polymerase was not altered by castration or injection of testosterone into the castrated animals (Figs. 3 and 4) (suggesting that the apparent template activity of this section of chromatin is not dependent on the androgenic status of animals). Although the NNF spectra of RNA synthesized by micrococcus polymerase on the purified rat DNA and that on the prostatic nuclear chromatin are very similar (Fig. 4), differences in NNF for certain dinucleotide sequences can be found in a reproducible manner.

Classification of prostatic nuclear chromatin into four categories: Studies in the last decade strongly support the concept that not all genes in a cell are equally expressed because not all the DNA can be indiscriminately transcribed. This has led to the recognition of the presence of an area of the nuclear chromatin which is inactive in carrying out transcription, while at another part of chromatin, RNA synthesis proceeds normally.[8] Among the so-called active areas of chromatin, the nucleolus-associated chromatin is often distinguished from the others, partly due to

TABLE 2

CLASSIFICATION OF PROSTATIC NUCLEAR CHROMATIN ACCORDING TO TEMPLATE PROPERTIES IN RNA SYNTHESIS AND ABILITY TO BIND ACTINOMYCIN D

Class	Regions*	Approximate % of total chromatin†	Ability to bind act. D	RNA Synthesis: By native polymerase C	By native polymerase T	RNA Synthesis: By added polymerase
I	M	70–80	−	−	−	−
II	R	20–30	+	−	−	+
III	Ch	1	+	+	+	+
IV	No	1	+	−	+	+

* Letters used to represent regions of chromatin may be read as M, masked; R, restricted or repressed; Ch, active chromatin; and No, nucleolar. These denotations are used for the convenience of discussion in this paper and are of course based on authors' conjecture.

† Figures given are based on the results of many experiments like that shown in Table 1 and Fig. 1.

the accumulating evidence that this part of the chromatin is responsible for the synthesis of ribosomal RNA precursors.[9]

From the foregoing findings one can visualize four classes of chromatin sections in the prostatic nucleus (Table 2). Class I, M regions, occupy 70–80 per cent of total nuclear DNA.[10] DNA in these regions of chromatin is physically masked and is not accessible to actinomycin D or RNA polymerase. Class II, R regions, occupy 20–30 per cent of total nuclear DNA. DNA in these regions of chromatin is available for actinomycin binding and can function as template in RNA synthesis by exogenous bacterial polymerase when the nucleus is ruptured. Prostatic nuclear RNA polymerase in the isolated nuclei does not appear to transcribe DNA of this region *in vitro*. Class III, Ch regions, occupy about 1 per cent of total nuclear DNA. DNA in these regions of chromatin can be transcribed *in vitro* by the RNA polymerase associated with prostatic nucleus. This RNA synthesis is not influenced by the levels of androgenic steroid in the experimental animals and is relatively insensitive to low concentrations of actinomycin D.[1, 2] Class IV, No regions, occupy about 1 per cent of total nuclear DNA. RNA synthesis at this region of chromatin *in vitro* is markedly enhanced (sevenfold) by the administration of testosterone to castrated rats and is extremely sensitive to a low concentration of actinomycin D *in vitro* or *in vivo*.[1, 2]

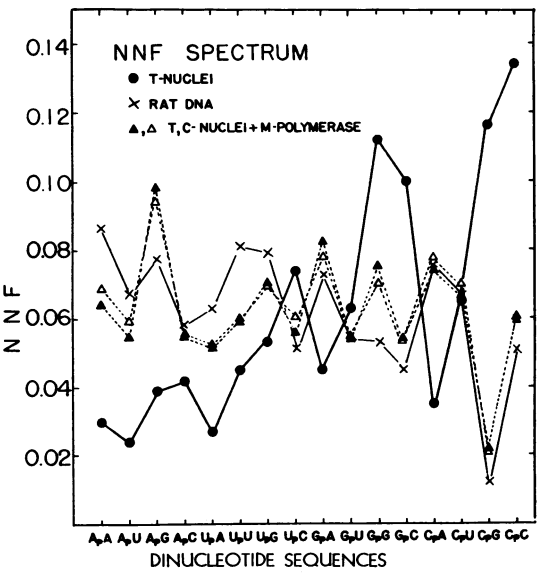

FIG. 4.—NNF spectrum of RNA synthesized by prostatic nuclei alone (●) or by pressure-disrupted prostatic nuclei (▲,△) and micrococcus polymerase. Treatment of animals was described in Fig. 3. T, C stand for nuclei from testosterone-treated and control castrates, respectively. For comparison, NNF for RNA synthesized by micrococcus polymerase and purified rat liver DNA (×) is also shown.

Androgen-induced selective transcription of DNA rich in dCpdG sequences: NNF for the CpG sequence of RNA transcribed from purified rat DNA is extremely low (0.012), whereas the same NNF for the same dinucleotide sequence of RNA synthesized by prostatic nuclei from animals injected with testosterone is surprisingly high (0.11). For further comparison, the NNF of RNA which can be made at each region of the DNA was calculated with several assumptions indicated in Table 3. The results show considerable variation in the CpG frequency of RNA made on each region of prostatic DNA, with the order of increase: M < R < Ch < No. Since the complementary nucleotide sequence in DNA for CpG is dCpdG, data tabulated in Table 3 suggest that dCpdG content in DNA of the No region, where androgen *in vivo* provokes selective enhancement of RNA synthesis, is about 24, 8, and 2 times, respectively, of that in DNA of M, R, and Ch regions. Other dinucleotide sequences, such as dCpdC, dGpdC, and dGpdG are also abundant in the No

TABLE 3

APPARENT NEAREST-NEIGHBOR FREQUENCIES OF RNA SYNTHESIZED AT DIFFERENT PARTS OF RAT PROSTATIC DNA*

	NNF				1/NNF			
Regions:	M	R	Ch	No	M	R	Ch	No
CpG	0.007	0.020	0.069	0.163	143	50	15	6
CpC	0.041	0.061	0.112	0.156	21	16	9	6
GpG	0.054	0.069	0.092	0.130	19	15	11	8
GpC	0.042	0.053	0.071	0.129	24	19	14	8
UpC	0.048	0.060	0.072	0.076	21	17	14	13
GpU	0.054	0.054	0.065	0.061	19	19	15	16
CpU	0.066	0.070	0.073	0.057	15	14	14	18
UpG	0.082	0.069	0.068	0.038	12	15	15	26
GpA	0.073	0.078	0.052	0.036	14	13	19	28
ApC	0.059	0.056	0.050	0.034	17	18	20	29
UpU	0.089	0.059	0.057	0.033	11	17	18	30
ApG	0.074	0.093	0.052	0.024	14	11	19	42
CpA	0.077	0.078	0.052	0.018	13	13	19	56
UpA	0.067	0.052	0.034	0.018	15	19	29	56
ApA	0.092	0.069	0.045	0.015	11	15	22	67
ApU	0.077	0.059	0.036	0.012	13	17	28	83

* NNF for R and Ch regions were obtained directly from experimental values. They were NNF of RNA made by pressure-disrupted prostatic nuclei from control castrated animals in the presence and absence, respectively, of micrococcus polymerase. NNF for RNA made by purified rat liver DNA and micrococcus polymerase was also obtained from direct experiments. NNF for RNA which could be made at M and No regions were calculated from above NNF values by assuming that M, R, Ch, and No regions occupy, respectively, 78, 20, 1, and 1% of total DNA and that, in the presence of testosterone *in vivo*, both Ch and No regions were transcribed.[10] 1/NNF shows the average number of nucleotides to be present in a nucleotide sequence which has one particular dinucleotide sequence in question.

region but since the concentrations of these sequences in other regions are not as low as dCpdG, the degree of "polarized distribution" of these sequences is not as remarkable as in the case of dCpdG.[10]

According to the calculations shown in Table 3, one can conclude that at No region of DNA, there is on the average one dCpdG for every six consecutive nucleotides, as compared to one in 143 at the M region. If the RNA codons in *E. coli* are universal and each dinucleotide sequence has an equal chance to be a 5′-OH terminal and middle nucleotide of each codon (two decisive nucleotides in a triplet codon[12]), RNA synthesized at this part of DNA could serve as messenger RNA for proteins rich in arginine (16%, CpG), proline (16%, CpC), glycine (13%, GpG), and alanine (13%, GpC). While such arginine-rich basic proteins may exist as histones or ribosomal proteins, one plausible view is that most of this RNA is in fact the ribosomal RNA precursor and does not function as a messenger RNA for other ordinary proteins.

Template activity of DNA associated with other regions of chromatin: In contrast to the No region, DNA in M, Ch, and R regions seems to have NNF and base composition compatible with a site for the synthesis of messenger RNA for ordinary proteins (Table 3). The existence of R regions presents some interesting problems. In the intact nuclei these regions may exist in a coiled (condensed) structure[13] and be accessible to actinomycin D but not to polymerase protein. When nuclei are disrupted, perhaps the DNA of this region is very efficient as a template in synthesizing RNA by exogenous (bacterial) polymerase. It is important to know whether any part of this region can participate in RNA synthesis *in vivo* at any given time of the cell life cycle, and if so, what regulatory mechanism is involved. Recently, several workers claimed to have evidence to show that several hormones enhance template activity of nuclear chromatin in target tissues.[14–17] These workers isolated chromatin from target tissues and tested its priming capacity in a bacterial

RNA polymerase system. In view of our present study, it is very desirable to know whether the chromatin isolated by the conventional procedure can represent quantitatively and qualitatively the entire nuclear chromatin (especially the relative proportion of each region).

The unavailability of DNA in M regions for either actinomycin binding or copying by RNA polymerase seems to suggest that this region (rich in adenine and thymine) is physically "masked" by histones. This suggestion is in agreement with the finding of several other workers[18-22] that the extent of inhibition by histones or other basic proteins on RNA synthesis *in vitro* by purified polymerase is greater with DNA higher in adenine and thymine.

Remarks on in vivo processes: The findings and discussion presented in this paper are largely based on experiments designed to analyze the effect of testosterone *in vivo* on the property for RNA synthesis *in vitro* by isolated prostatic nuclear preparations. Further study is required to see whether such *in vitro* experiments reflect nuclear RNA synthesis *in vivo*. For example, the relationship between the RNA synthesized *in vitro* by isolated nuclei and that actually formed *in vivo* and believed to be retained only inside the nuclei[23, 24] should be clarified.

The mechanism by which testosterone enhances the synthesis of RNA at a selective and small section of chromatin is exquisite (we have recently found that 17β-estradiol, within 4 hr after injection to immature rats, can also cause a similar stimulatory effect on the synthesis of certain type of RNA (actinomycin D sensitivity and changes in NNF)[25]). Even if an increase in active polymerase protein[26] is responsible for the increase in the RNA-synthesizing activity, another mechanism is required to maintain a high level of active RNA polymerase at the selective and small region of chromatin defined as No region (presumably nucleolar) in this paper. While a repressor-derepressor theory[27] is attractive, other mechanisms are not unlikely. For example, this may be simply due to sequestering (by membrane) of this region of chromatin and the physical restriction on transport or synthesis of polymerase at this part of cell nucleus. The changes in the chemical (e.g. ionic) environment due to an hormonal effect on the "membrane"[28] of this compartment may play an important role in regulating RNA polymerase activity. Moreover, the specific association of polymerase and DNA template may involve a "conformational fit" of molecules required for the initiation of polynucleotide formation and the restricted distribution of certain nucleotide sequences may serve an important function in directing polymerases to a specific part of the DNA template or regulating the rate of RNA synthesis.

Summary.—Prostatic nuclear chromatin can be classified in four categories according to its capacity for the synthesis *in vitro* of different species of RNA by DNA-dependent RNA polymerases. M regions occupy about 70–80 per cent of the total nuclear DNA, and DNA at this region is not accessible to actinomycin D or RNA polymerase. R regions occupy about 20–30 per cent of total nuclear DNA. This DNA is available for the binding of actinomycin and can function as template for RNA synthesis by additional bacterial polymerase when nucleus is ruptured. Ch regions have about 1 per cent of total nuclear DNA. RNA synthesis at this region of intact nucleus continues even after animals are deprived of androgens. Only a very small portion (1% or less) of nuclear DNA is associated with No regions where androgen *in vivo* provokes selective enhancement of RNA synthesis. Nearest-

neighbor frequency study suggested that the nucleotide sequence in the DNA of each region is unique. The deoxycytidylyl(3′,5′)deoxyguanosine sequence in the prostatic DNA appears to have a striking nonrandom distribution. The apparent content of this base sequence in DNA of No regions was about 24, 8, and 2 times, respectively, of that in DNA of M, R, and Ch regions.

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