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Hormonal control of gene expression: differential activation of rat bone marrow RNA polymerases by erythropoietin and testosterone*

Control hormonal de la expresión génica: Activación diferencial de las RNA polimerasas de médula ósea de rata por eritropoyetina y testosterona

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ERYTHROPOIETIN TESTOSTERONE BONEMARROW
MESSENGER RNA RIBOSOMAL RNA HEMOGLOBIN

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Hormones play a role in the regulation of gene expression by inducing changes in enzyme patterns in target cells mediated by the synthesis of specific RNA molecules. Erythropoiesis has been used as a system for studying the molecular mechanism of regulation of gene action by means of two hormones: erythropoietin and testosterone. Erythropoietin triggers the erythropoietic process acting on the erythropoietin sensitive cells (ESC), in which the hormone induces the synthesis of a high molecular weight RNA, which is the precursor of a functional 9 S messenger RNA. Testosterone seems to act on polychromatophilic erythroblasts, in which the synthesis of ribosomal RNA or its precursor is stimulated. The steroid enhances the nuclear ribonuclease activity, which could represent a control mechanism for the processing (maturation) of high molecular weight RNAs.

The incorporation of ^{3}H-GTP and ^{3}H-UTP into RNA bv isolated rat bone marrow nuclei is stimulated by erythropoietin and testosterone. Using α-amanitine and differente ionic strength conditions it was found that erythropoietin enhances preferentially RNA polymerase II activity while testosterone increases RNA polymerase I activity.

It is postulated that erythropoietin and testosterone act synergically to create the biochemical machinery for hemoglobin synthesis, the macromolecule that characterizes the erythropoietic process.

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In living cells the expresion of genetic information involves selective transcription of DNA into RNA molecules. These, in turn, participate in the synthesis of proteins. In mammalian cells, and in general, in eucaryotics cells, the regulation of these processes comprises the participation of several mechanisms, among them, the action of hormones seems to represent an important step in the control of gen expression.

The rate of synthesis of specific RNA molecules and/or the levels of enzyme activities are assessed as a function of hormones. In this way, it is possible to correlate appearance of some RNA types with the creation or the stimulation of the biochemical machinery for the synthesis of specific enzymes that characterize the metabolic behaviour of cells.

Generally, the RNAs initially produced by gene transcription are synthesized as pre-RNAs, which are compounds of high molecular weight, not identical to the functionally competent, mature forms of RNA. The RNA precursors are converted to the mature forms by specific molecular reactions known as processing.

The three major stages in the expression of genetic information are: transcription, processing and function. Each stage represents potential regulation site in which several hormones may be acting.

A suitable biological model to study the biochemical action of hormones is the erythropoietic process, where the synthesis of hemoglobin represents the main feature of the phenomenon. Erythropoiesis is a gradual process of cell differentiation and proliferation leading from stem cells to highly differentiated cells: the erythrocytes (1, 2, 3).

The glycoproteic hormone erythropoietin (EP) is the central factor initiating and regulating erythropoiesis and, thus may control the synthesis of hemoglobin. The hormone is recognized by the erythropoietin-sensitive-cells (ESC), derived by differentiation from the stem cells by an already unknown mechanism, inducing the differentiation of these kind of cells to erythroblasts in which the synthesis of hemoglobin occurs (4, 5).

The initial action of EP is its binding to an specific membrane receptor. The external EP signal is then transduced in intracellular message that reaches the cell nucleus where a molecular effect on the genome is produced. The whole recognition mechanism is not well understood and accordingly to Goldwasser et *al* (6, 7), it represents a sensitization process by which cells synthesize EP receptors; thus, ESC can be defined as erythropoietic cells with such receptors.

When the EP "message" reaches the chromatin, the first molecular event described is the stimulation of the synthesis of several RNA types. Within 5 min of EP administration, rat marrow cells synthesize a large RNA not found in control animals. Gross and Goldwasser, *in vitro* (8) have described this RNA with a sedimentation coefficient around 150S, while we have reported RNA species in the range 93 to 85S in *in vitro* experiments. It is assumed that this high molecular weight RNA represent the precursor of functional RNA for the erythropoietic process and it seems that after processing, it gives rise to different RNAs with intermediate sedimentation coefficients up to 9S RNA, being this sort of RNA required for globins synthesis (9).

It has been shown that testosterone and other androgens stimulate erythropoiesis. The stimulatory effect of androgens on the metabolism of bone marrow cells may be interpreted through two mechanism: an early and direct one, acting on some particular bone marrow cells, related to the erythropoietic line and a late and indirect one mediated by an increase in the amount of endogenous erythropoietin (10).

In our laboratory, Minguell *et al.* (10) have demonstrated that testosterone enhances the synthesis of nuclear RNA. The sedimentation analysis of nRNA showed that the hormone stimulated preferentially the incorporation of labelled precursor into species with sedimentation coefficients higher than 30S. Among these species, the ribosomal RNA precursors, with sedimentation coefficients in the range of 45S and bearing nucleolar origin, should be present.

The above observations strongly suggest that EP and testosterone are acting at the transcription stage, but in a different way. While EP seems to induce the synthesis of a pre-messenger RNA, testosterone activates the synthesis of pre-ribosomal RNA (1, 2).

To asses the possibility that EP and testosterone stimulate the synthesis of different

RNA types, the activity of RNA polymerases were tested in *in vitro* studies. Testosterone enhances preferentially RNA polymerase I activity in rat bone marrow cells synthesizing r RNA. In spleen from plethoric mouse, Piantodosi *et al* (11) found that at early times (30 min) EP stimulates the activity of RNA polymerase II, while at 3 h later, the activity of RNA polymerase I is enlarged.

Besides the effects on RNA polymerases activities and RNA synthesis, testosterone augments the activity of specific endonucleases, establishing another level of regulation in the control mechanism on the processing of high molecular weight RNAs (10, 12, 13).

The work reported here, extends these observations in order to examine further relationships between the action of both EP and testosterone at transcription level. We have measured in rat bone marrow cells the effects of both hormones on the activity of RNA polymerases I and II and on the synthesis of RNA.

MATERIALS AND METHODS

a) *Biological materials*

Female Sprague-Dawley rats (180-240 g) were used for the RNA polymerase experiments, while male Wistar rats (250-260g) were used for the RNA analysis experiments. Anemie was produced by 3 subcutaneous injection of 1 mg per 10g weight animal of phenylhydrazine chlorhydrate neutralysed solution in 3 days intervals. Polycythemia was induced by intraperitoneally injection of 10 ml suspension of isologous heparinized blood cells. Hemoglobin was determined by the method of Cannan (15).

b) *Biochemical materials*

Erythropoietin was prepared from anemic rabbit plasma as previously described (16). The biochemical reagents were obtained from Sigma Chemical Company, Saint Louis Mo. USA and from Calbiochem, La Jolla, California, USA. ^{3}H-uridine ^{3}H-GTP and ^{3}H-UTP were purchased from New England, Nuclear, Boston, USA.

c) *RNA experiments.*
I) *Density gradient analysis*

RNA was basically isolated following the phenol method described by Georgiev, with some modifications (2, 16). The RNA samples were analyzed on a sucrose-density gradients (5-20%), in buffer Tris-HCl pH 7,4 with 1 mM EDTA; 10 mM NaCl and 0,1% (w/v) diethylpyrocarbonate. The gradients were run for 4h at 38.000 rpm in a SW 39 rotor. Forty fractions (0.12 ml each) were collected, diluted with 1 ml of H_2O and the absorbance at 260 nm was measured. The sedimentation coefficients were calculated with the McEwen Tables (17); the computed values agreed well with the sedimentation prolifes obtained with the E. coli 23S RNA. The radioactivity was measured with a liquid scintillation spectrometer (Nuclear Chicago Mark I) using a mixture of 4 g PPO, 400 ml ethanol and 600 ml toluene as a scintillator with an efficiency of 30%. Proper blanks were run for each determination.

II) *RNA polymerase experiments*

From pooled femurs and tibias derived from 4 to 6 animals, bone marrow cells were removed and washed with ice-cold saline solution. The preparation of the nuclear fraction were performed as previously described (10). The resulting nuclear pellet was suspended in 60 mM Tris-HCl buffer pH 8.1. Aliquots were taken for measurement of RNA synthesis and for DNA content (18).

RNA synthesis was measured at low ionic strength in a reaction mixture containing 60 mM Tris-HCl buffer, pH 8.1; 5 mM $MgCl_2$; 30 mM KCl; 10 mM mercaptoethanol; 0.1 mM GTP, ATP and CTP, 2 uCi ^{3}H-UTP or ^{3}H-GTP and a proper volume of the nuclear suspension (100-150 μg DNA) in 0.5 ml. For assays performed at high ionic strength the mixture also contained 0.4 M ammonium sulphate and 5 mM $MnCl_2$.

Reaction (15 min at 37°C) was terminated by the addition of 5 ml of 10% (w/v) trichloroacetic acid containing 1 mM sodium pyrophosphate. Mixtures were chilled and yeast RNA (200 μg in 0.2 ml) was added as carrier. The precipitate was separated by centrifugation and the RNA was then isolated by a method already described (19). In some experiments, the precipitate obtained was filtered on nitrocellulose filters (HAMP, Millipore), washed (5 ml × 4) with 10% TCA and dried. The samples radioactivity was counted, as it was mentioned above.

In all the experiments, RNA synthesis was estimated in terms of counts per minute or desintegration per minute of radioactive precursor incorporated into RNA or into acid-insoluble material per 10 μg DNA.

RESULTS AND DISCUSSION

1. ACTION AT TRANSCRIPTIONAL STAGE

a) *Effect of erythropoietin and testosterone on RNA synthesis*

We have suggested that EP can exert a discriminatory action on the genome, inducing the synthesis of RNA with messenger characteristcs (16, 20). However, other investigators (3, 9, 21) have communicated the activation of the synthesis of several RNA species by the hormone. The results obtained depend on the biological system and principally, on the use of different methods for the isolation of RNA.

In Fig. 1 it is shown the effect of EP on the synthesis of rat bone marrow RNA, at different times. The profiles obtained at 15 min may

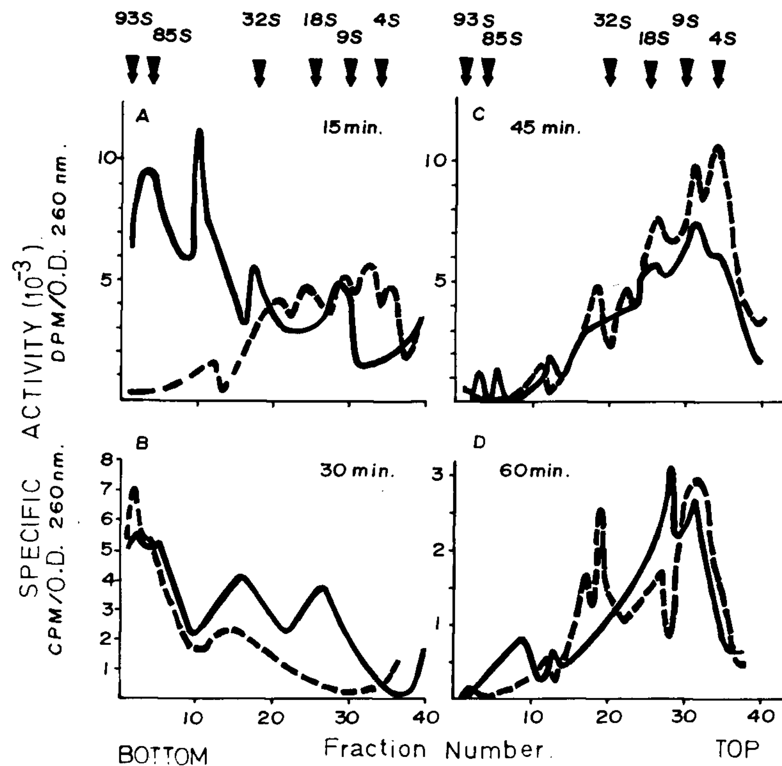

Fig. 1. Sucrose density-gradient analysis of total RNA isolated from control rat bone marrow cells and erythropoietin-treated cells.

Bone marrow cells were incubated at 37°C at different times (15, 30, 45 and 60 minutes) in a medium containing buffer TRIS-HCl pH 7.4; 0.25 M sucrose; 5 mM glucose; 1 M $MgCl_2$ and 15 μCi ^3H-uridine. 10 units of erythropoietin were added to the experimental tubes. The continuos line represents control and the dotted line erythropoietin-treated cells.

Other conditions are indicated in the text.

reflect the activation of processing mechanisms or the slightly stimulation in the synthesis of RNAs in the range 9 to 4S. At 30 min, EP induces the synthesis of high molecular weight-RNA (93 to 85S). The effect is very conclusive because the profiles of the control and treated cells are different, specially at 15 and 30 min. It seems clear that at 45 and 60 min after EP RNAs in the range 18 to 4S appear. The high specific activity of the RNAs may signify the synthesis of these RNA types or may be a consequence of processing.

In order to demonstrate the rate of RNA labelling, radioactivity of some RNA species was plotted against time, as shown in Fig. 2. The 93S and 85S RNA had a rapid turnover in comparison to the other RNA, being both stimulated by the EP. The 32S and 18S RNA had an slight decrease in the incorporation of ^{3}H-uridine and they were not activated by the EP, meanwhile 9S and 4S RNA had a tremendous increase in its activities starting at the 30 min, being higher than the RNA stimulated by the EP. If we compare the figures of 93 and 85S RNA with 9 and 4S RNA, the data resembles typical precursor-products relationships.

These findings strongly suggest that 93S and 85S RNA may be the precursors of 9S and 4S RNA being the product of the induction provoked by the EP. Meanwhile the 32 and 18S RNA species not affected by the EP, seem to correspond to ribosomal RNA.

Minguell *et al.* (10) have shown that testosterone increases the activity of RNA with sedimentation coefficients larger than 30S. Among these species, probably the ribosomal RNA precursors with sedimentation coefficients in the range 45 to 30S with a nucleolar origin are present. Valladares *et al.* (22) have confirmed these results after partial charac-

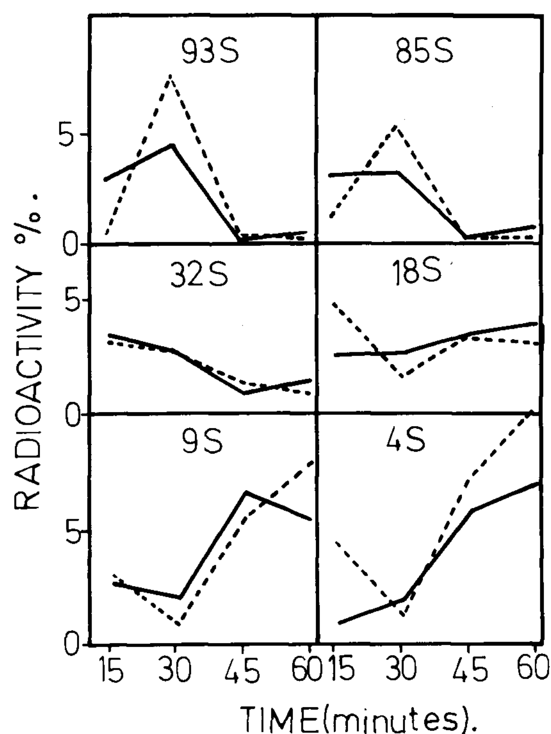

Fig. 2. Rate of RNA labeling. The radioactivity % from the peaks of RNA are plotted against time. The values were obtained from Fig. 1. Solid line is control and broken line is erythropoientin-treated cell.

terization by density gradient analysis of the RNA synthesized in the presence of testosterone. The centrifugation patterns of RNA, extracted from bone marrow nuclei, showed that among the stimulated RNA species are those presenting sedimentation coefficients within 14-30S.

The main postulate that can be drawn from these observations is that EP selectively stimulates the transcription of high molecular weight RNAs, probably preformed DNA-like RNA, while testosterone specifically activates the synthesis of ribosomal RNA species.

b) *Effect on the activity of nuclear DNA-dependent RNA polymerases*

DNA-dependent RNA polymerases activity accounts for the synthesis of almost all the RNA species of cell nucleus. The regulation of RNA polymerase activity by hormones provides an enzymatic basis to explain the origin of several RNA types and it seems to represent a common property of several hormones (23, 11, 24).

Eukaryotic cells contain at least three classes of RNA polymerase whose enzymatic characteristics, intranuclear location, divalent cation and ionic strength-requirements and sensitivity to inhibition by α-amanitine, are well known (23).

We have reported (19) that EP increases RNA polymerase activities in rat bone marrow, when it is injected intravenously to the animal. However, we were not able to identify the specific action of the hormone on each of the three kinds of polymerases known today.

We performed experiments in isolated rat bone marrow nuclei, in order to establish the effects of EP and testosterone on RNA polymerase activity. In table I the ^{3}H-GMP incorporation into the RNA of isolated bone marrow nuclei obtained from normal, polycythemic

TABLE I

RNA polymerases activities from normal, polycythemic and anemic rats.
Effect of α-Amanitine (α A)

Conditions	*DPM ^{3}H-GMP incorporated/ 10 μg DNA*
Normal	23.190 ± 1.920
Normal + α - A	2.940 ± 250
Polycythemic	15.600 ± 1.380
Policythemic + α - A	1.800 ± 90
Anemic	29.100 ± 2.540
Anemic + α - A	2.750 ± 181

Model experiment. It was carried out at high ionic strength. The figures indicate the mean of 3 samples ± S.E. Hemoglobin percent of the rats were as following: normal: 14-16, polycythemic: 19-21 and anemic: 6-7. Other experimental conditions are indicated in the text.

and anemic rats is shown. The experiments were done at high ionic strength and in the presence of 10 ug of α-amanitine. Different patterns were obtained under the 3 experimental conditions: under polycythemic conditions in which the erythropoietic process is almost suppressed, the activity of RNA polymerase was decreased, while in anemic animals, in which erythropoiesis is very augmented, the activity of the enzyme was increased, compared with normal rats. There was 90% of inhibition after administration of α—amanitine in the three conditions above described.

The results in table II show that at high ionic strength ^{3}H-GMP incorporation was 40% larger, in EP treated animals, as compared with controls, while no change was observed in testosterone-treated animals. Polymerase II activity is optimal at high ionic strength in the presence of Mn^{++} and it is sensitive to low concentrations of α-amanitine. The principal localization of this enzyme is in the nucleoplasm and it might be involved in the synthesis of heterogeneous nuclear RNA, a part of which is presumably the precursor of messenger RNA.

TABLE II

The effect of erythropoietin, testosterone and α-amanitine (αA) on RNA synthesis by rat polycythemic bone marrow at high ionic strength

Conditions	*DMP ^{3}H-GMP incorporated / 10 μg DNA*
Control	11.820 ± 1.035
+ α-A (10 μg)	8.550 ± 433
+ erythropoietin	14.430 ± 1.738
+ erythropoietin + α-A	7.200 ± 440
+ testosterone	11.160 + 1.020
+ testosterone + α-A	8.370 ± 986

Model experiment. The figures indicate the mean of 3 samples ± S.E. erithropoietin (4.5 units/rat) and testosterone (250 μg/rat) were injected intravenously 3 hours before sacrifice. Other conditions are similar of those of table I.

TABLE III

The effect of testosterone and α-amanitine (α-A) on RNA synthesis by isolated bone marrow nuclei at low and high ionic strength (lis and his)

Conditions	*DPM ^{3}H-UMP incorporated / 10 μg DNA*	
Control	7.490 ± 820	36.070 ± 3.150
+ testosterone	11.100 ± 1.200	43.100 ± 4.200
+ α-A (0.1 μg/ml)	5.900 ± 420	28.090 ± 1.500
+ α-A (10 μg/ml)	4.800 ± 300	4.960 ± 200

Model experiment. The figures indicate the mean of 3 samples ± S.D. Other experimental conditions are the same of those of table II.

The effects of testosterone and α-amanitine on RNA synthesis by bone marrow nuclei at low and high ionic strength, are shown in table III.

A 5 fold increase in polymerase activity is observed at high ionic strength, in comparison with low ionic strenght. Testosterone stimulates ^{3}H-UMP incorporation up to 50% in low ionic strength, while in the other condition the incorporation increases only in a 20% in comparison with the controls. When bone marrow nuclei are incubated at the two ionic strength in the presence of two concentrations of α-amanitine, there is a notorius inhibition in the ^{3}H-nucleotide incorporation into RNA at high α-amanitine concentration in high

ionic strength medium, while in the other condition only reflect 20-30% of inhibition.

Polymerase I activity is optimal at low ionic strength in the presence of Mg^{++} and it is resistant to α-amanitine. The preferential nucleolar localization of this enzyme may suggest that it could be responsible for the synthesis of ribosomal RNAs.

The ability of EP and testosterone to increase the activity of multiple forms of RNA polymerase, indicates that the molecular action of both hormones is at some level in the transcription stage. The selective effect of testosterone on the activity of the nucleolar Mg^{++}-dependent RNA polymerase I and the discriminatory action of EP on the activation of the nucleoplasm Mn^{++}-dependent RNA polymerase II indicates that both hormones might act at different biochemical levels in the genome.

The results above discussed support our previous findings in whose we have postulated a synergistic mechanism to regulate the system for hemoglobin synthesis (1, 2, 25).

2. *Action at post-transcriptional stage*

The RNAs transcript are large molecules which must be processed to functional forms by specific reactions catalyzed by endonucleases.

There is scarce information about the regulatory action of hormones at this level. In our laboratory, Sierralta *et al* (10) have found that after the administration of testosterone to female rats, a 30% increase in the free nuclear RNase activity was achieved. They suggested that free RNase is necessary for an acceleration of the processing rate of these RNA precursors into functional RNAs. In other work, Sierralta and Minguell (13) have reported a marked decreased of 47 to 50% of the enzymatic activity of total ribonuclease activity in the nuclear fraction derived from polycythemic rat bone marrow cells when it was compared with the nuclear ribonuclease activity of normal rat bone marrow cells.

In mammalian cell RNA processing includes cleavage of RNA precursors and selective degradation of the non-conserved portions. In the case of messenger RNAs the formation of 5' terminal cap structures and 3' terminal poly A segments and the methylation of particular internal adenine residues may represent an important step in the regulation of protein synthesis (26).

In the special case of globins m RNAs it was suggested that regulatory controls are present at steps following transcription of globins mRNAs or mRNAs precursor (27) It has been established that 5'-capping is important for globin m RNA binding to ribosomes (9) and the addition of poly A to the 3' OH terminal chain of the globin m RNA may represent other fundamental stage in the synthesis of globin.

Regulation at the level of processing has the advantages of responding to a variety of external stimuli and of insuring the fidelity of genetic information transfer (28).

3. *Action at cytological level*

In spite of the considerable amount of understanding that has been obtained from the origin of hematopoietic cells, there is scanty information available regarding the molecular mechanism that controls the differentiation process (4).

It is well known that the pluripotent stem cells give rise to committed precursos cells, erythropoietin responsibe cells (ERC) or erythropoietin sensitive cells (ESC), which in turn are committed precursors of erythroid line cells (5).

Erythropoietin is the factor that induces the differentiation of ERC to early proerythroblasts.

Testosterone and its metabolites stimulate erythropoiesis, but the exact cytological level where androgenic hormones act, is still not clearly understood and has been the subject of controversy (14). Some evidences appear to indicate that the β-H-metabolites of androgens induce cycling of pluripotential stem cells which thus provides a growing fraction of ERC.

Preliminary morphologic studies of bone marrow cells and the myeloid: erythroid ratio in polycythemic rats treated with methyltestosterone indicated an increased in the amount of polychromatic and basophylic normoblasts and a variation of the ratio from 16 to 2. This shift is due to the augmentation of erythroid cells and not a state of reduction of myeloid serie cells (2).

CONCLUSIONS

The data presented in this paper, strongly suggest that erythropoietin and testosterone exert their genetic action at the transcriptional level to effect the synthesis of nascent messenger RNA and precursor ribosomal RNA, respectively, which in turn, may conform the biochemical system to synthesize the globins.

Figure 3 presents a model in which we propose a molecular mechanism of EP and testosterone action on erythropoiesis. The model is based in data assembled by several groups, including ours, studying the effects of these hormones.

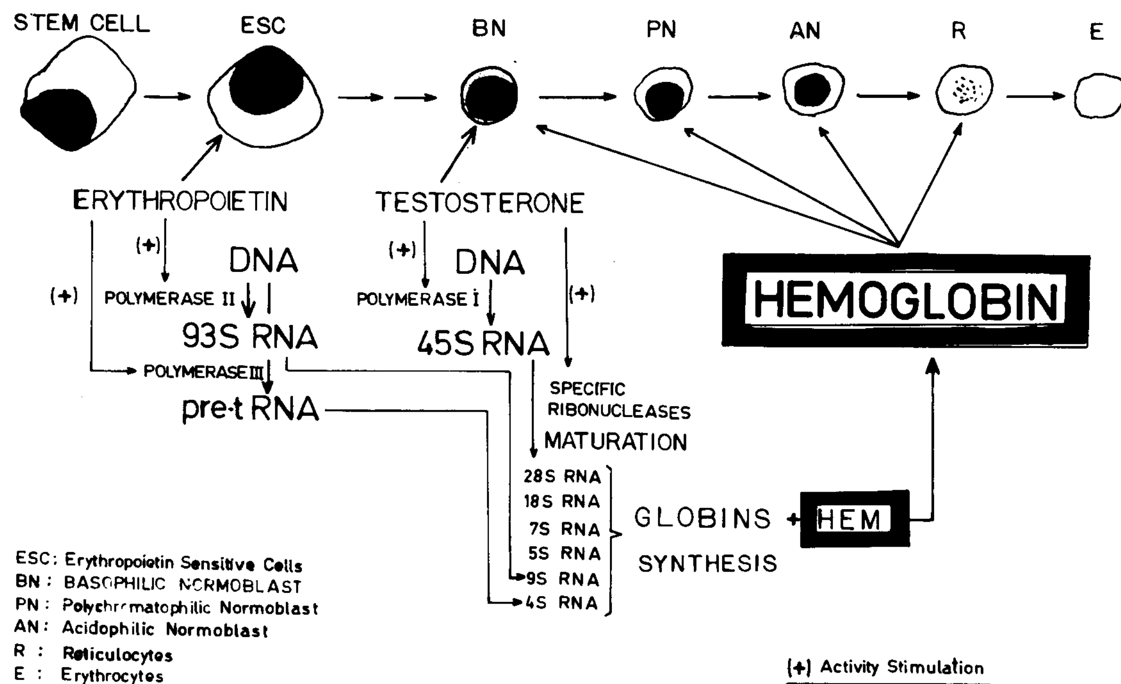

Fig. 3. Proposed model for the synergic molecular mechanism of action of erythropoietin and testosterone.

The model indicates the following:

I. Both hormones act at transcriptional level.

II. Both hormones interact with the genome in different way. Erythropoietin induces the synthesis of high molecular weight RNA (93 to 85S) which may be the precursors of the functional 9S RNA. It is not clear its molecular participation in the synthesis of transfer RNA which is stimulated after to EP action.
Testosterone stimulates the synthesis of ribosomal RNA precursors.

III. Erythropoietin increases the activity of DNA-dependent RNA polymerase II while testosterone augments the activity of DNA-dependent RNA polymerase I.

IV Both hormones seem to act at different cytological levels: EP on the ERC cells and testosterone on the polychromaphylic erythroblasts.

V. The idea of a synergic effect of both hormones on erythropoiesis at molecular level is supported by the findings of this paper.

RESUMEN

Las hormonas participan en la regulación de la expresión génica, induciendo cambios del espectro enzimático en células blancos al sintetizar RNAs muy específicos.

La eritropoyesis constituye un buen sistema biológico para el estudio de los mecanismos moleculares de la regulación de la actividad del gen y en él participan las hormonas eritropoyetina y testosterona.

En este trabajo se presentan experimentos destinados a comprobar la interrelación bioquímica de ambas hormonas en la regulación del proceso.

Ambos factores parecen actuar a diferentes niveles tanto citológicos como bioquímicos.

Eritropoyetina gatilla el mecanismo eritropoyético actuando sobre las células sensitivas a su acción (ESC), induciendo la síntesis de un RNA de elevado peso molecular, que se considera precursor de uno funcional reconocido como el RNA mensajero 9S.

Testosterona parece actuar sobre los eritroblastos policromatofílicos en los cuales estimula la síntesis de RNA ribosomales o de sus precursores. El esteroide aumenta la actividad de ribonucleasas nucleares, mecanismos que puede representar la regulación del procesamiento o maduración de RNAs de alto peso molecular a RNAs funcionales más pequeños.

La incorporación de ^{3}H-GMP y ^{3}H-UMP a RNAs de núcleos aislados de médula ósea de rata es estimulada por ambas hormonas en forma separada. Utilizando α-amanitina y condiciones a distintas fuerzas iónicas, se encontró que la eritropoyetina estimula preferentemente la actividad de la RNA polimerasa II, mientras que la testosterona aumenta la actividad de la RNA polimerasa I.

Se postula que la eritropoyetina y la testosterona actúan sinérgicamente para generar la maquinaria bioquímica que sintetiza la hemoglobina que es la macromolécula que caracteriza el proceso.

SUMMARY

Hormones play a role in the regulation of gene expression by inducing changes in enzyme patterns in target cells mediated by the synthesis of specific RNA molecules.

Erythropoiesis has been used as a system for studying the molecular mechanism of regulation of gene action by means of two hormones: erythropoietin and testosterone.

Experiments designed to correlate the biochemical action of both hormones on rat marrow cells are herein reported.

Both factors seems to act at different biochemical and citological levels.

Erytropoietin triggers the erythropoietic process acting on the erythropoietin sensitive cells (ESC), in which the hormone induces the synthesis of a high molecular weight RNA, which is the precursor of a functional 9S messenger RNA.

Testosterone seems to act on polychromatophilic erythroblasts, in which the synthesis of ribosomal RNA or its precursor is stimulated. The steroid enhances the nuclear ribonuclease activity, which could represent a control mechanism for the processing (maturation) of high molecular weight RNAs.

The incorporation of ^{3}H-GMP and ^{3}H-UMP into RNA by isolated rat bone marrow nuclei is stimulated by erythropoietin and testosterone. Using α-amanitine and different ionic strength condition it was found that erythropoietin enhances preferentially RNA polymerase II activity while testoterone increases RNA polymerase I activity.

It is postulated that erythropoietin and testosterone act synergically to create the biochemical machinery for hemoglobin synthesis, the macromolecule that characterizes the erythropoietic process.

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