Effects of Testosterone on Qualitative Pattern of Protein Synthesis in Skeletal Muscle*

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ABSTRACT: The patterns of protein synthesis in intact soleus muscle from testosterone-treated and control castrated rats were compared by disc electrophoresis and isoelectric focusing in polyacrylamide gels. No appreciable differences in the labeled proteins could be detected under conditions in which both ribonucleic acid and protein synthesis were stimulated 60% by the steroid hormone. Comparison of these with our earlier results (which had demonstrated a similar increase in template activity of muscle chromatin following injection of testosterone) leads us to suggest that the transcriptional effects of testosterone in skeletal muscle involve activation of redundant genes or increased expression of previously active genes. These results do not allow a choice between possible regulatory and structural roles of the genes activated by testosterone. Our studies on testosterone effects in muscle demonstrate that large increases in template activity of chromatin do not necessarily indicate a corresponding increase in the kinds of protein synthesized by the tissue.

Our earlier studies on cell-free preparations from muscle hormone-treated rats led us to suggest that testosterone caused a substantial increase in the template activity of DNA in muscle chromatin (Breuer and Florini, 1966). This could allow more rapid synthesis of mRNA, which in turn could account for the increased activity of ribosomes isolated from muscle of castrated rats following injection of testosterone (Breuer and Florini, 1965). Thus we concluded that the anabolic effect of testosterone resulted largely if not entirely from a "derepression" of DNA in muscle genes.

One aspect of the results with chromatin was rather disturbing. Chromatin isolated from castrated rats 16–24 hr after injection of testosterone propionate was 60–80% more active than chromatin from control rats. If this increase in template efficiency resulted in the synthesis and translation of many new kinds of mRNA (thus accounting for the increased activity of the ribosomes isolated from testosterone-treated rats), then substantial changes in the structure and/or function of muscle could be expected. In fact, except for a general increase in size, no changes in muscle of treated rats could be detected. The present studies were undertaken to determine whether this gross observation was a valid indication of the effect of testosterone at the molecular level, *i.e.*, did androgen administration cause any detectable changes in the qualitative pattern of protein synthesis in muscle? The results of a series of double-label experiments indicate that disc electrophoresis and isoelectric focusing do not reveal any differences in the population of proteins synthesized in muscle following administration of testosterone. Thus an increase in the template activity of chromatin does not necessarily indicate synthesis and translation of a greater number of mRNA species; the additional DNA sequences made available upon testosterone administration may be redundant copies of genes already active in castrated rats, may be involved in regulatory processes, or may even bear no direct relationship to the testosterone induced increase in protein synthesis in muscle.

Materials and Methods

[^{14}C]Leucine (278 mCi/mmole) was purchased from New England Nuclear Corp. [^{3}H]Leucine (12 Ci/mmole) was prepared by reduction of Δ^{4}-leucine as described previously (Florini, 1964). Testosterone propionate was purchased from Calbiochem. Apparatus and reagents for disc electrophoresis were from Canalco; ampholites used in isoelectric focusing were from LKB. NCS and other counting supplies were purchased from Amersham-Searle.

Rats were normal male albino Sprague–Dawley rats from Russell Miller Farms, Cazenovia, N. Y. They were castrated 6–7 days before hormone injection, and were supplied food and water *ad libidum* throughout the experiment. Testosterone propionate was injected as a microcrystalline suspension at 0.5 mg/ml in 0.5% (w/v) carboxymethylcellulose. Each rat received 0.1 mg of testosterone propionate by intraperitoneal injection 24 and 16 hr before zero time; controls received 0.2 ml of carboxymethylcellulose solution at the same times. At zero time, rats were put under deep ether anesthesia and soleus muscles were dissected out, care being taken to avoid cutting fibers in this muscle.

Isolated soleus muscles were incubated in 5 ml of Krebs–Ringer bicarbonate buffer containing either [^{3}H]leucine (20 μCi/ml) or [^{14}C]leucine (4 μCi/ml). In addition, the incubation medium contained 180 mg % glucose and 19 amino acids (omitting leucine) at the concentrations found by Scharff and Wool (1965) in rat plasma. Muscles were incubated 2 hr at 37° with shaking at 90 cycles/min under an atmosphere of 5% CO_2 and 95% O_2. All solutions were thoroughly gassed with this mixture just prior to addition of the muscle.

Two rats were used for each experimental group. In all cases, one soleus from a rat was incubated with [^{14}C]leucine

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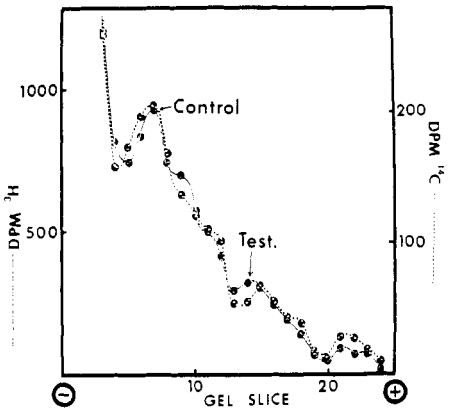

FIGURE 1: Disc electrophoresis (1 hr) of muscle-soluble proteins. Labeled proteins from soleus muscles of testosterone-treated (solid line) and control (dotted line) castrated rats were separated by electrophoresis at 5 mA/gel column for 1 hr. Procedures for labeling of proteins and the preparation and analysis of gels are described under Materials and Methods.

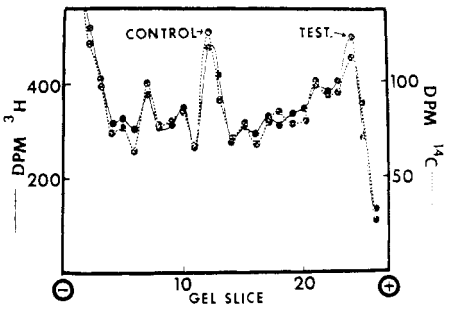

FIGURE 2: Disc electrophoresis (3 hr) of muscle-soluble proteins. All conditions and designations are as described under Figure 1, except that the gels were run for 3 hr rather than 1 hr.

and the other with [^{3}H]leucine. At the end of the incubation period, muscles were removed from the buffer, blotted, and combined as follows: ^{3}H control with [^{14}C]treated and *vice versa*. These mixed muscles were then homogenized (Kontes conical all-glass homogenizer) in 3 ml of homogenizing medium (Breuer *et al.*, 1964) and centrifuged at 30,000 rpm in the SW-39 rotor of a Spinco Model L ultracentrifuge. The pellet was washed carefully and suspended in 3 ml of 9 M urea and stored overnight at room temperature. The soluble fraction was dialyzed overnight at 4° against two changes of 4000 ml of 0.01 M Tris (pH 8.0). The protein solution was then added to quantities of solid sucrose and urea weighed to give a final concentration of 6 M urea and 20% (w/v) sucrose; these samples were exposed to 6 M urea at least 1 hr prior to electrophoresis. A portion of the dialyzed soluble proteins was set aside for isoelectric focusing; no urea or sucrose was added to this sample.

Soluble proteins were analyzed by disc electrophoresis by the method of Davis (1964), except that sample and stacking gels were omitted and the protein samples (in 20% sucrose) were applied directly to the top of the separating gels. The Canalco Model 66 apparatus and procedure were used; all solutions contained 6 M urea. Residue proteins were analyzed by electrophoresis in 9 M urea as described by Florini and Brivio (1969). Isoelectric focusing was done by the technique described by Catsimpoolis (1969). In all cases, gels were removed from the tubes, frozen in Dry Ice–ethanol, and sliced with a block of razor blades held together with stainless steel bolts and separated by 1.5- or 0.7-mm Teflon spacers. The gel slices were incubated in glass counting vials in 0.2 ml of NCS at 60° for 2 hr; the vials were tilted so that this volume of NCS was sufficient to cover the slices. After this incubation, 10 ml of toluene phosphor (4 g/l. of 2,5-diphenyloxazole, 50 mg/l. of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] was added, and the vials were placed in a rotary shaker for 1 hr. Radioactivity was determined in a Nuclear-Chicago Unilux II three-channel liquid scintillation counter equipped with an external standard. Window and attenuator settings were arranged to exclude ^{3}H from the B channel; under the conditions of these experiments, ^{3}H was counted at approximately 25% efficiency and ^{14}C at 65% efficiency. The counter was calibrated for variation of counting efficiency with external standard channels ratio over a wide range of counting efficiencies; quadratic equations were fitted to ten-point calibration curves for ^{3}H in the A channel and for ^{14}C in both the A and B channels. Data from each sample were analyzed to determine channels ratio, counting efficiencies, and disintegrations per minute for each isotope. These rather tedious calculations were greatly facilitated by use of a Programma 101 computer.

Results

Amino Acid Incorporation into Protein by Soleus Muscles in Vitro. Preliminary experiments were conducted to establish the properties of the amino acid incorporating system in intact soleus muscles. Under the conditions of these experiments, incorporation continued actively for more than 2 hr and was linear for at least 60 min. Addition of cycloheximide at 0.1 mg/ml caused 95% inhibition of leucine incorporation. In general, approximately 2×10^6 dpm of [^{3}H]leucine and 4×10^5 dpm of [^{14}C]leucine were incorporated into protein in each soleus muscle; in all cases, muscle from testosterone propionate treated rats was 50–70% more highly labeled than that from corresponding control castrated rats.

Disc Electrophoresis of Soluble Proteins. Figures 1 and 2 illustrate typical results of disc electrophoresis of muscle soluble proteins after 1- and 3-hr electrophoresis in 6 M urea at 5 mA/column. In the short period, much of the protein did not move into the gel, but we considered this analysis necessary to investigate possible effects of testosterone on the synthesis of small or highly charged proteins. Longer electrophoresis gave more satisfactory migration of the proteins. However, neither set of conditions revealed substantial differences in labeling of protein by muscle from treated compared with control rats. (Preliminary experiments in which [^{3}H]- and [^{14}C]-leucine were incubated together revealed that variations of approximately 5% can be expected in apparent labeling of proteins analyzed by our counting and computing procedures.) In all cases, similar results were obtained when proteins labeled by reversed assignment of isotopes were analyzed by disc electrophoresis. We have now done six separate experiments in which patterns of labeling were examined by these techniques; in no case was any significant difference in the pattern of protein synthesis detected.

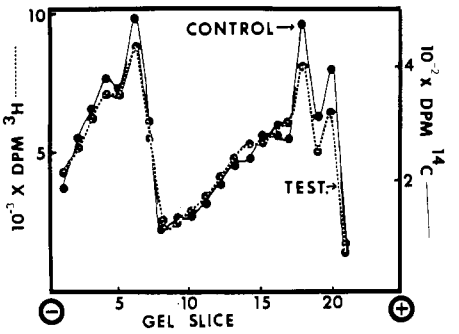

FIGURE 3: Disc electrophoresis of muscle residue proteins in 9 M urea. The pellets from 100,000*g* centrifugation of soleus muscle of testosterone-treated (dotted line) and control (solid line) castrated rats were solubilized in 9 M urea and analyzed by disc electrophoresis in 9 M urea as described by Florini and Brivio (1969). Procedures for labeling of proteins and analysis of gels are described under Materials and Methods.

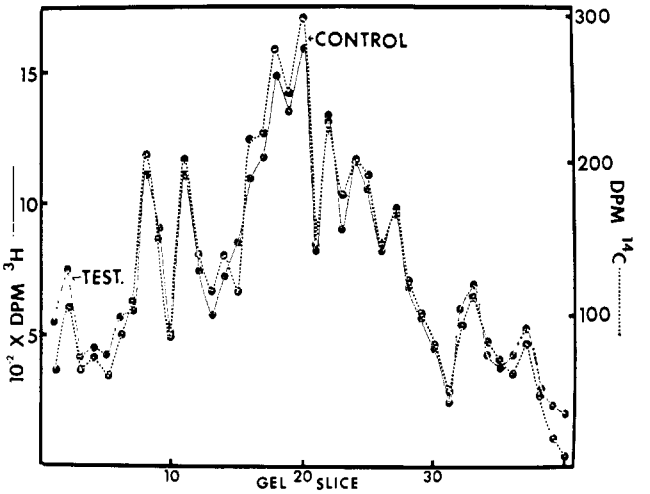

FIGURE 4: Isoelectric focusing of muscle-soluble proteins. Labeled proteins from soleus muscles of testosterone-treated (solid line) and control (dotted line) castrated rats were separated by isoelectric focusing in pH range 3–10 by the technique of Catsimpoolis (1969). Procedures for labeling of proteins and analysis of gels are described under Materials and Methods.

Disc Electrophoresis of Residue Proteins. We (Florini and Brivio, 1969) have devised a procedure for disc electrophoresis of myosin in dilute polyacrylamide in the presence of 9 M urea. Figure 3 presents typical results of analyses of muscle pellet proteins solubilized in 9 M urea. Although quantitative limitations prevented us from including myosin markers in these gels, our experience with this system allows us to suggest that the large peak at the left corresponds to the large subunit of myosin; the more rapidly moving peaks may be either nuclear or mitochondrial proteins or small subunits of myosin. As in the case of the soluble proteins, there was no apparent difference in labeling of residue proteins from androgen-treated compared with control rats in six experiments, each of which involved analysis of two sets of doubly labeled proteins.

Isoelectric Focusing of Soluble Proteins. While these studies were in progress, techniques for analytical isoelectric focusing in polyacrylamide gel columns came to our attention. This procedure provides sensitive analysis of protein mixtures, depending upon properties somewhat different from those which control separation in disc electrophoresis, so it was used to analyze proteins in the last two of our experiments. The procedure of Catsimpoolis (1969) gave very striking resolution of the complex mixture of muscle soluble proteins, as is illustrated in Figure 4. Even under these conditions, in which at least 12 distinct peaks could be observed, no differences in labeling of muscle soluble proteins could be detected in muscle from testosterone-treated rats.

Evaluation of Methods and Tissue Response. The consistent observations of identity of labeled proteins led us to question whether our techniques might simply be inadequate to separate different proteins effectively. Consequently we conducted several comparisons of labeled proteins which we could expect to be different. In one instance, a mixture of ^{3}H-labeled muscle proteins and ^{14}C-labeled liver proteins was compared by disc electrophoresis for 3 hr; at least six peaks could be detected in proteins from one tissue but not the other. A similar analysis of labeled proteins from young (75 g) *vs.* mature (350 g) rats by isoelectric focusing revealed three areas of substantial difference among the 10–12 major peaks in the gel. Thus we are satisfied that our procedures can detect differences in protein populations when such differences actually exist.

Although soleus muscles comprised a part of the population of muscles used in our earlier studies on effects of testosterone administration on RNA and protein synthesis in skeletal muscle, it is conceivable that this muscle is unresponsive to testosterone and thus makes no additional proteins when the steroid is injected into the animal. The small size of the muscle (approximately 30 mg in our animals) precluded isolation and assay of ribosomes and nuclei, but it was possible to measure the incorporation of labeled precursors into RNA and protein. As mentioned above, the incorporation of leucine into protein was increased 50–70% in soleus muscles from testosterone-treated compared with control rats. Similar experiments using labeled uridine as precursor indicated that RNA synthesis was increased 60% under the conditions of our injections and incubations. Of course, these increases in precursor incorporation may result from changes in intracellular pools or in rate of transport of the labeled precursor, but it is reassuring that the magnitude of the increases corresponds closely to that observed previously in cell-free preparations (Breuer and Florini, 1955, 1966) in which transport and precursor dilution could not affect the results.

Discussion

Superficial examination of the tissue indicates that androgen stimulation of muscle growth does not cause any major change in the structure or function of skeletal muscle. Our current results indicate that this is also true at the level of protein synthesis; there are no major qualitative changes in synthesis of muscle proteins following injection of testosterone propionate. When these results are compared to those we obtained in studies of RNA polymerase and chromatin from androgen-treated rats, there is an apparent discrepancy. More DNA is available for transcription, more RNA and protein are synthesized, but no additional kinds of protein can be detected. It would seem logical for a gross nonspecific increase in protein

synthesis to occur at the translational rather than the transcriptional level of gene expression.

There are several possible explanations for this apparent discrepancy. The most simple is that our procedures are not sensitive enough to reveal the presence of additional proteins synthesized in response to testosterone. However, it seems unlikely that a 50–70% qualitative change in the protein population could be missed by methods with such high-resolving power, and control experiments demonstrated that differences in other protein populations were readily detected by our procedures.

It is also possible that many additional RNA molecules are synthesized but are not translated into protein; the increase in protein synthesis could conceivably be an independent translational effect of testosterone. Our earlier studies (Breuer and Florini, 1965) did not clearly demonstrate a specific translational effect of testosterone; the observed differences in activity of ribosomes could be attributed to differences in mRNA content of the ribosome preparations. It is possible that there were also effects on the inherent activity of individual ribosomes, but the experiments were not designed to provide a sensitive assay of such effects.

In our opinion, the most likely explanation of our results is that most if not all of the additional DNA made available for transcription repeats sequences which are already transcribed in muscle of castrated rats. This redundancy might occur at the level of the integrator or producer genes postulated by Britten and Davidson (1969); in either case, increases in amount but not kinds of proteins synthesized would result.

Evaluation of these possibilities now requires detailed qualitative analysis of the RNA synthesized in muscle of testosterone-treated rats. Although we have been attempting such analyses for some time, thus far we have been unable to achieve sufficient labeling of muscle RNA to allow useful qualitative analyses. However, some progress is now being made, and our efforts are continuing; preliminary observations indicate that there are no major qualitative changes in muscle RNA synthesis following administration of testosterone propionate to castrated rats.

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References

Breuer, C. B., Davies, M. C., and Florini, J. R. (1964), *Biochemistry 3*, 1713.
Breuer, C. B., and Florini, J. R. (1965), *Biochemistry 4*, 1544.
Breuer, C. B., and Florini, J. R. (1966), *Biochemistry 5*, 3857.
Britten, R. J., and Davidson, E. H. (1969), *Science 165*, 349.
Catsimpoolis, N. (1969), *Biochim. Biophys. Acta 175*, 214.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Florini, J. R. (1964), *Biochemistry 3*, 209.
Florini, J. R., and Brivio, R. P. (1969), *Anal. Biochem. 30*, 358.
Scharff, R., and Wool, I. G. (1965), *Biochem. J. 97*, 257.

Regulation of the *in Vivo* Synthesis of the Polypeptide Chain Elongation Factors in *Escherichia coli**

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ABSTRACT: The rate of protein synthesis is constant per ribosome as the bacterial cell adjusts the relative concentrations of supernatant proteins to ribosomes at different growth rates. In this investigation, the regulation of the synthesis of the polypeptide chain elongation factors T and G was studied. *In vitro* activity measurements, polyacrylamide disc electrophoresis, and immunochemical assays were used to determine the relative levels of the polypeptide chain elongation factors in cells in different steady states of growth. It is concluded that the relative amount of factors to ribosomes stays constant at the different growth rates, and that the factors are present in the cell at *ca.* 1 mole of each/mole of ribosomes.

In a defined steady state of growth, bacterial cells have a unique macromolecular composition (Schaechter *et al.*, 1958; Neidhart and Magasanik, 1960; Kjeldgaard and Kurland, 1963; Ecker and Schaechter, 1963; Maaløe and Kjeldgaard, 1966). It seemed of interest to determine whether (1) the synthesis of the polypeptide chain elongation factors T and G (Nishizuka and Lipmann, 1966) was coordinated with that of the ribosomes in different steady states, since both form part of the protein synthetic machinery, or (2) whether they behaved as total cellular proteins (Maaløe and Kjeldgaard, 1966). The relative amount of total cellular protein to ribosomes increases with increasing generation times (Kjeldgaard and Kurland, 1963; Ecker and Schaechter, 1963). It is now found that under the same conditions, synthesis of the polypeptide chain elongation factors is coordinated with that of the ribosomes and not with the total cellular

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