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CHANGES IN HEPATIC ANDROGEN SENSITIVITY AND GENE EXPRESSION DURING AGING

BANDANA CHATTERJEE[1] and ARUN K. ROY[1,2]

Departments of [1]Cellular and Structural Biology and [2]Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, TX 78284, U.S.A.

Summary—The hepatic tissue of the male rat exhibits a gradual decline and ultimate loss in androgen responiveness during *in vivo* aging. Appearance of the age-associated androgen insensitivity can be delayed by dietary calorie restriction, an effective means for life-span extension. The androgen receptor mRNA is detectable in the liver only in its androgen-responsive state. Pubertal appearance of hepatic androgen sensitivity is remarkably correlated with the concomitant appearance of a cytoplasmic androgen binding (CAB) protein. Androgen resistance during senescence is associated with the loss of hepatic CAB activity as well. We are investigating the molecular basis for the temporal modulation of this hormone sensitivity through studies on the differential expression of two androgen-responsive marker genes. These are the androgen-repressible SMP-2, and the androgen-inducible α_{2u}-globulin. Androgen resistance of hepatocytes during aging results in repression of the α_{2u}-globulin gene, and derepression of the SMP-2 gene. The structural organizations for both of these genes have been characterized. The role of nuclear transcription factors (androgen receptor and any other transacting factor(s) which may be involved) in the coordinate regulation of α_{2u}-globulin and SMP-2 during aging and nutritional manipulation is being explored to establish the molecular mechanism of andropause in the liver.

INTRODUCTION

Aging is a multicomponent process, under the control of genetic as well as epigenetic factors [1]. The species-specific fixed mean life-span potential of organisms predicts that a genetically determined program plays a fundamental role in both development and aging. Physiological aging is thus an extension of the continuum of specific gene repression and derepression which are hallmarks of embryogenesis, development and maturation. The programmed life-span potential of an organism is, however, altered to a certain extent by environmental, nutritional and hormonal factors, just as embryogenesis, development and growth can be potentially disrupted by teratogens and hormonal imbalance. A cumulative challenge of genetic and non-genetic factors on the regulation of certain key gene products may initiate a cascade effect on many other gene products. This eventually results in a myriad of time-dependent changes at molecular, cellular and systemic levels. A combinatorial effect of the resulting phenotypic changes manifest as aging.

Hormone receptors are one class of key regulatory elements which play pivotal roles in the maintenance of structural and functional integrity of cells. Aging is commonly associated with a gradual dysfunction (primarily down-regulation) of sex hormone receptors resulting in an ultimate arrest of the reproductive potential of organisms [2]. Similar to reproductive maturation and senescence of the ovary, androgen responsiveness of the rat liver undergoes discrete and almost abrupt changes at maturation and during senescence [3, 4] In our laboratory, we have been investigating the molecular basis for this temporal influence on hepatic androgen sensitivity, and the consequent differential expression of androgen-responsive genes in the liver. Our studies on specific gene expression during *in vivo* aging reflect physiological changes of a combinatorial nature, i.e. they account for the accumulated pleiotropic effects of both genetic and non-genetic processes.

HEPATIC ANDROGEN SENSITIVITY AND ITS TEMPORAL MODULATION

During the course of maturation and aging, androgen sensitivity of the rat liver is demarcated by three distinct phases of hormone

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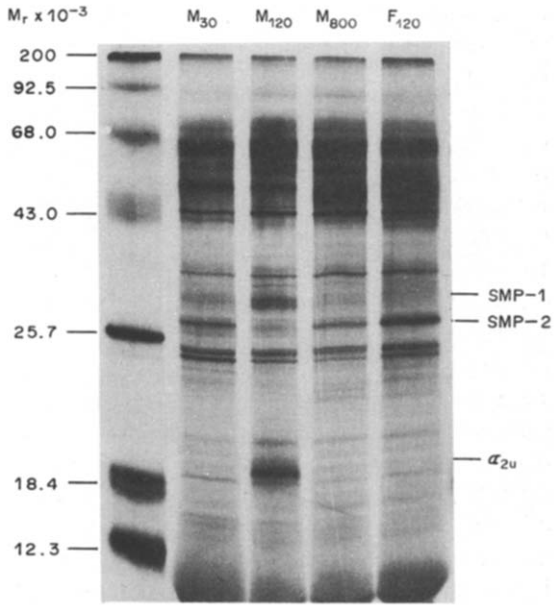

Fig. 1. Effects of age and sex on the hepatic mRNA expression. The autoradiogram represents electrophoretically separated *in vitro* translation products of total hepatic mRNAs from male rats of ages 30 day (M_{30}), 120 day (M_{120}), 800 day (M_{800}) and the female rat of 120 days (F_{120}). The molecular weight markers are displayed on the extreme left lane. Equivalent amounts of radioactive proteins were applied in each lane.

sensitivity, namely, prepubertal androgen insensitivity, androgen responsiveness during adulthood, and subsequent androgen resistance during senescence. Only during the androgen-responsive state, hepatocytes can respond to androgenic hormones, and synthesize α_{2u}-globulin and senescence marker protein-1 (SMP-1), two androgen-inducible proteins [5]. On the same token, the synthesis of senescence marker protein-2 (SMP-2), an androgen-repressible protein, is drastically inhibited during this period [5]. The autoradiographic picture in Fig. 1 demonstrates the effects of age and sex on the mRNA expression for α_{2u}-globulin, SMP-1 and SMP-2 in the liver. The diametrically opposite modes of androgenic regulation result in a coordinate repression and induction of the SMP-2 and α_{2u}-globulin genes. During the periods of androgen insensitivity, the androgen-repressible SMP-2 mRNA, which is detected only at a low level in the adult male, is expressed at a relative high abundance in prepubertal and senescent males. Androgen insensitivity also results in the constitutive expression of the SMP-2 mRNA in the female liver. In a coordinate regulation, time-dependent repression and derepression of the SMP-2 gene are inversely correlated with the transcriptional induction of the androgen-inducible α_{2u}-globulin in the post-pubertal male liver, and its repression during senescence (>750 days of age). It is of interest to point out that even under supra-physiological doses of androgen, the hepatic tissues of prepubertal and senescent male rats remain resistant to the androgenic induction of α_{2u}-globulin [3].

With regard to changes in hepatic androgen sensitivity, our recent observation on the androgen receptor mRNA level during prepuberty, adulthood and senescence is particularly important. Using the 5′ hypervariable end of the receptor cDNA sequence as the hybridizing probe, we have clearly demonstrated the presence of the androgen receptor mRNA in the adult male liver. The receptor mRNA is non-detectable in prepubertal (<40 days of age) and senescent (>750 days of age) male rats, and in the female of all ages. Thus, the loss of androgen sensitivity in the liver is the direct result of the loss in the androgen receptor mRNA.

Studies in our laboratory have identified a 31 kDa cytoplasmic androgen binding (CAB) protein in the adult male rat liver [3, 6]. CAB can be detected only during the androgen responsive state of the liver. Animals such as prepubertal, senescent and androgen-sensitive mutant (testicular feminized) males lack this binding activity. CAB is assayed either through sucrose density gradient analysis of the liver cytosol prelabeled with radioactive dihydrotestosterone (DHT) or by SDS–PAGE of the cytosolic proteins following their photoaffinity labeling with radioactive R 1881, a synthetic androgen. This low-abundant (50 fmol/mg total protein), high affinity ($K_d = 10^{-8}$ M) binding protein is highly androgen-specific as shown in Table 1. Quantitative analysis shows a strong correlation between the appearance and disappearance of CAB and the hepatic

Table 1. Specific binding of [^{3}H]R 1881 to adult (100-day-old) male liver cytosol and competition by nonandrogen steroids[a]

Competing steroid	Displacement (%)
R 1881	100
Triamcinolone acetonide	6.2
Progesterone	5.8
Estradiol-17β	31.1

[a]Binding assays were performed in the presence of 58 nM [^{3}H]R 1881 and a 500-fold molar excess of unlabeled competing steroids. In the absence of any unlabeled competing steroids, 52.0 fmol of R 1881 was specifically bound to 1 mg of cytosolic protein (100% binding). From Ref [6].

Table 2. Correlation between age- and sex-specific binding of [^{3}H]R 1881 to liver cytosol and level of hepatic α_{2u}-globulin[a]

Liver cytosol	Specific R 1881 binding (fmol/mg of protein)	Cytosolic α_{2u}-globulin (ng/mg of protein)
Adult male (100 days)	52.98	240.00
Adult female (100 days)	6.53	0.85
Immature male (30 days)	5.14	0.41
Old male (850 days)	7.99	2.66

[a]Values are means of three animals for each age group From Ref. [6].

synthesis of the androgen-inducible α_{2u}-globulin (Table 2).

We have purified CAB from the adult male rat liver cytosol via a combination of separation procedures including salt fractionation, ion-exchange chromatography and denaturing polyacrylamide preparative gel electrophoresis [7]. Use of the CAB-specific polyclonal antibody in Western blot analyses has shown that the triphasic changes in the hepatic CAB activity during maturation and aging, as determined through androgen-binding assays, remarkably correlate with corresponding changes in the levels of immunoreactive CAB (Fig. 2). At present the relationship between the 31 kDa CAB and the 76 kDa rat androgen receptor is unknown. To this end we are considering the following three possibilities: (1) CAB is a proteolytic fragment of the androgen receptor; (2) CAB is a product of alternate splicing of the androgen receptor gene; (3) CAB is distinct from the androgen receptor, but it belongs to the superfamily of ligand binding proteins of nuclear hormone receptors.

In an intriguing observation, we have reported that the androgen-insensitive state of the liver (i.e. during prepuberty and senescence in the male and in females of all ages) correlates with the presence of a 29 kDa weak androgen binding protein [6]. The polyclonal antibody raised against the 31 kDa CAB does not react with the 29 kDa binding component. Elucidation of the molecular relationship between these two androgen binding proteins may provide important clues to the mechanistic rationale of programmed changes in androgen sensitivity during aging.

AGE-ASSOCIATED CHANGES IN SPECIFIC GENE EXPRESSION IN THE LIVER

We examined the effect of age on specific protein synthesis by hepatocytes grown in short-term primary cultures [8]. Hepatocytes, derived from male rats of progressively advancing age, were radiolabeled with [^{35}S]methionine, and the individual hepatic proteins were identified as well-resolved radioactive spots on the 2-dimensional gel electrophoretogram. The results in Fig. 3 show that aging is associated with decreased rates of synthesis for some proteins (indicated as black circles in Figs 3A and B) and increased rates of synthesis for some other proteins (depicted with white circles). A catalogue of different proteins, along with their isoelectric points and relative molecular mass, showing either increased or decreased rates of synthesis during transition from young adulthood (150 days of age) to middle age (450 days of age), and from early senescence (600 days of age) to late senescence (750 days of age), has been described [9]. The multiple isoelectric forms of α_{2u}-globulin appear as a cluster of radioactive spots around the 18.4 kDa molecular weight region in the autoradiogram derived from the 150-day-old male rat. Identity of these protein spots as corresponding to α_{2u}-globulin

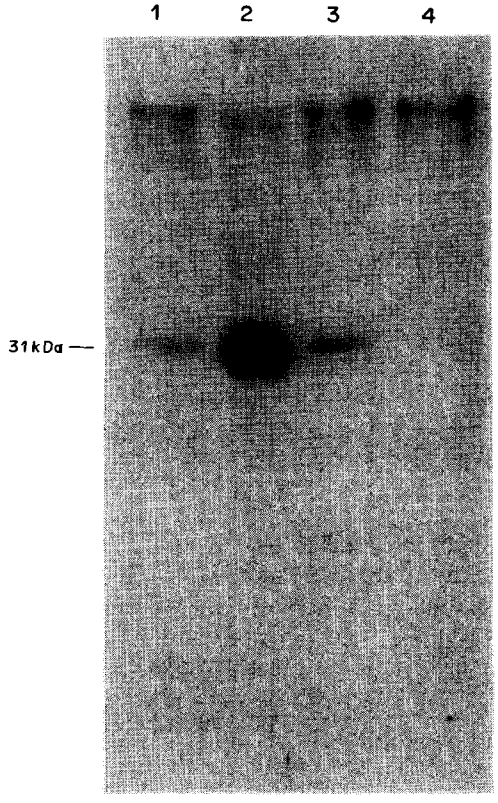

Fig. 2. Age-dependent changes in hepatic levels of the immunoreactive CAB as analysed by Western blotting. Lanes are marked as follows: (1) prepubertal male (30-day); (2) young adult male (100-day); (3) senescent males (850-day); (4) adult female (100-day). 30 mg of cytosolic proteins were applied in each lane. The 31 kDa protein band in the immunoblot is marked.

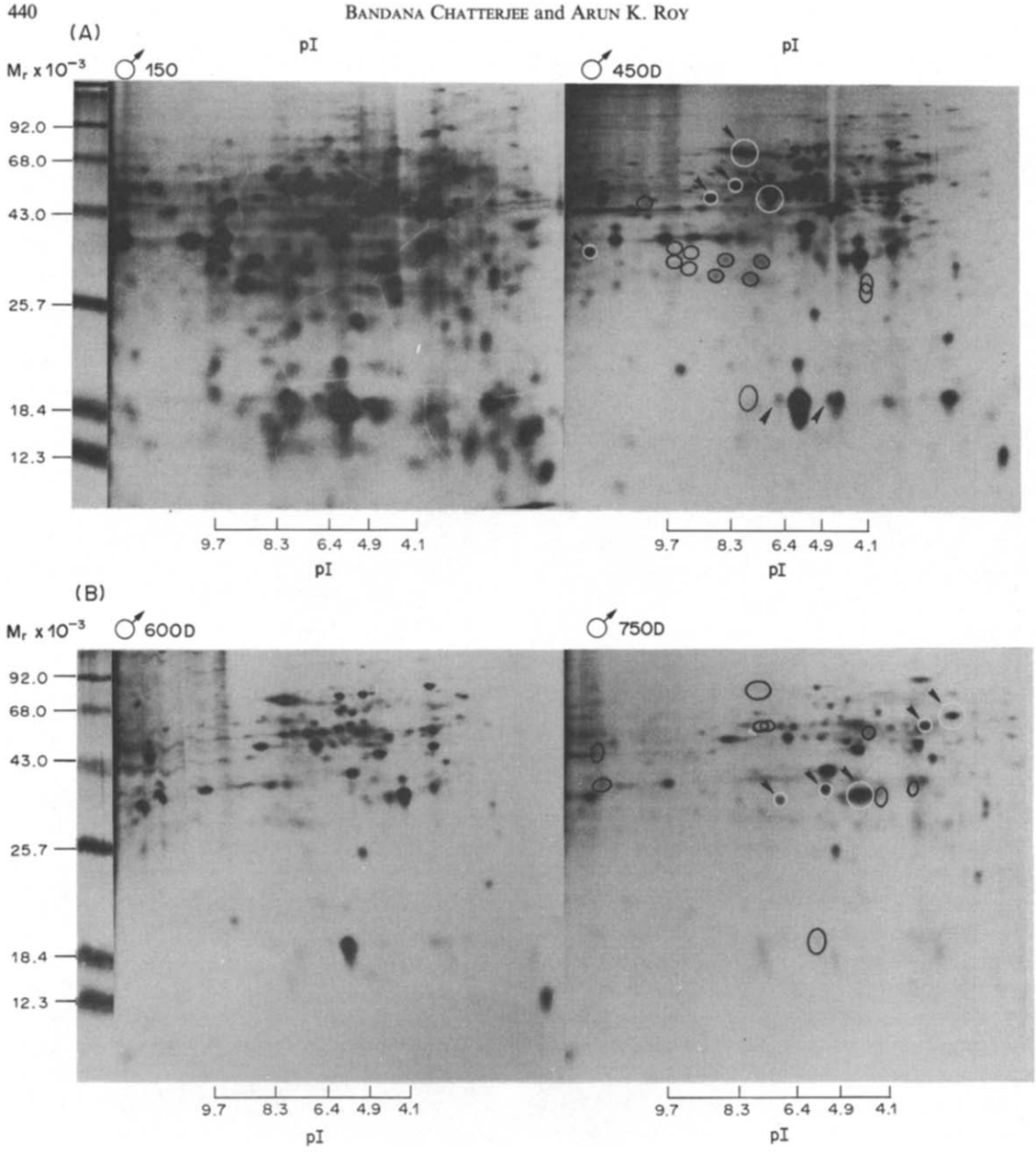

Fig. 3. Age-dependent changes in specific protein synthesis by male rat hepatocytes pulse labeled with [^{35}S]methionine. Comparative autoradiograms derived from (A) 150-day-old male vs 450-day-old male hepatocytes and (B) 600-day-old male vs 750-day-old male hepatocytes.

has been established by virtue of their specific recognition by the monoclonal antibody to α_{2u}-globulin [10]. Some of the isoelectric forms of α_{2u}-globulin cease to be synthesized as early as 450 days of age. By 750 days, none of the α_{2u}-globulin isoforms are detectable. The cluster of proteins around 34–36 kDa regions, which show augmented synthesis during transition from early to late senescence may represent SMP-2. Their identity has not been however confirmed.

The genes for SMP-2 and α_{2u}-globulin have been cloned [11–15], and currently we are investigating the role of DNA (*cis*-regulatory element)–protein (*trans*-acting element) interactions in the androgen-modulated regulation of these two proteins. Our assumption is that changes in the expression of these hormone-responsive genes are mediated by an altered activity of the androgen receptor which acts as a nuclear transcription factor. Furthermore, an age-dependent change in specific trans-acting factors other than hormone receptors can conceivably influence the altered gene expression.

α_{2u}-Globulin is encoded by a family of 25–30 highly homologous (less than 5% divergence in the nucleic acid sequence) genes within the

Table 3. Transcriptional rates of the α_{2u}-globulin gene during aging

Age of the male animal (days)	α_{2u}-Globulin gene transcription (specific hybridizable dpm per 6×10^6 dpm of total RNA transcripts)	Transcriptional activity compared to the young adult (%)
30	30 (36, 24, 31)	2.5
92	1200 (1192, 1222, 1188)	100
425	683 (663, 670, 718)	56
760	76 (63, 67, 100)	6.3

From Ref. [17].

haploid genome. These genes are clustered on chromosome 5. The mouse homolog of rat α_{2u}-globulin is known as MUP, or mouse urinary protein. Individual MUP genes are organized within a 720 kb stretch of the chromosomal DNA [16]. The α_{2u}-globulin gene domain in neonatal and in very young (<20 days of age) rats is organized within a highly compact chromatin area so that the gene is inaccessible to digestion by deoxyribonuclease (DNasel). At around 3 weeks of age a major reorganization of the chromatin structure for α_{2u}-globulin genes renders α_{2u}-globulin DNA sequences readily sensitive to DNasel digestion. Such an "open" configuration of the gene domain is maintained for the rest of the life of the animal. As described earlier, α_{2u}-globulin gene transcription in the rat liver begins only at the onset of puberty (around 40 days of age). The gene induction coincides with the appearance of the 31 kDa CAB protein. Transcriptional activation of the α_{2u}-globulin gene is associated with attachment of this gene domain to the nuclear matrix. Senescence-associated transcriptional inactivation of α_{2u}-globulin is accompanied by matrix detachment of this gene [17]. Age-dependent regulation of α_{2u}-globulin gene expression has been reviewed extensively [18]. More recently we have isolated the SMP-2 gene and characterized its age-dependent regulation [12]. The results of nuclear run off studies summarized in Tables 3 and 4 demonstrate the changes in both α_{2u}-globulin and SMP-2 gene expression during aging primarily reflect corresponding changes in the rates of transcription of these genes.

TISSUE-SPECIFIC AND ANDROGEN-REPRESSIBLE EXPRESSION OF SMP-2

The geomic clones for SMP-2 have been isolated from a rat genomic library. Extensive characterization of the isolated clones have revealed that SMP-2 is encoded by at least two genes which are distinct, yet highly homologous [12]. Both of these genes are expressed in the liver during its androgen insensitive state. SMP-2 gene expression is greatly reduced in the androgen-responsive adult male liver. The predominant site of SMP-2 synthesis is the liver, although an extremely low, albeit detectable level, of SMP-2 is synthesized also in the prostate. Within 24 h of castration, SMP-2 mRNA expression in the prostate is markedly enhanced.

In order to characterize the regulatory domain of the SMP-2 gene responsible for its liver-specific expression, we have evaluated the promoter functionality of the upstream sequence of this gene in rat hepatoma cells through transfection assays. Using one of the isolated SMP-2 genes, we prepared a hybrid CAT construct in which the SMP-2 upstream sequence (−1970 to +38 bases) was linked to the downstream CAT (chloramphenicol acetyltransferase) gene. A partial map of this hybrid

Table 4. Transcriptional rates of the SMP-2 gene during aging[a]

Age of male (M), female (F) rats (days)	SMP-2 gene transcription (specific hybridizable dpm per 6×10^6 dpm of total RNA transcripts)	Transcriptional activity compared to the young adult female (%)
30 M	820 (820, 840, 800)	54.12
150 M	313.3 (350, 300, 290)	20.66
800 M	1050 (1000, 1100, 1050)	69.30
150 F	1516.6 (1500, 1510, 1540)	100

[a]Averages from three experiments were used to compute percent normal female, and individual values are given within parenthese. The background radioactivity (binding of labeled nuclear RNAs to the non-recombinant pBR322 DNA immobilized on the nitrocellulose paper) was subtracted from the individual dpm values. The transcriptional rates of the constitutive expression of the albumin gene in the liver do not vary during aging (Ref. [12]).

construct is shown in Fig. 4A. Upon gene transfection, this plasmid construct drives the expression of the reporter CAT gene in the rat hepatoma cells FTO2B, but not in the fibroblast-derived mouse L cells (Fig. 4B). Studies with additional constructs carrying progressively deleted SMP-2 promoter sequence from the 5′ direction show that at least 600 bases of the upstream sequence of the SMP-2 gene is required for its liver-specific expression.

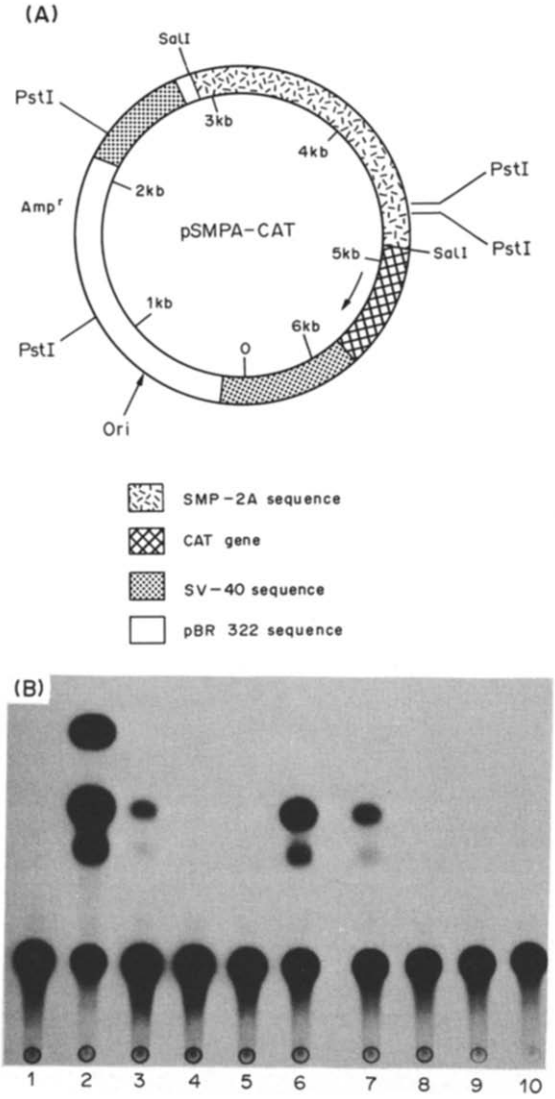

Fig. 4. Liver-specific promoter activity of the SMP-2 gene determined through DNA transfection and subsequent assay of the reporter CAT activity. (A) Restriction map of the hybrid CAT construct used in the transfection study. The numbers indicate distance in kilobases. (B) CAT activities of transfected cells. Lanes 3–6: FTO2B cells (rat hepatoma). Lanes 7–10: La$^-$tk$^-$ cells (mouse fibroblast derived cell lines). 1: mock transfection. 2: mock transfection plus commercial CAT enzyme. 3 and 7: pSV2-CAT transfection (positive control). 4 and 8: transfection of parent vector (negative control). 5 and 9: transfected with a hybrid plasmid which has the SMP-2 promoter linked to the CAT gene in a reverse orientation. 6 and 10: transfection of the experimental plasmid containing the SMP-2 promoter (−1970 to +38 bases) linked to the CAT gene in a parallel orientation.

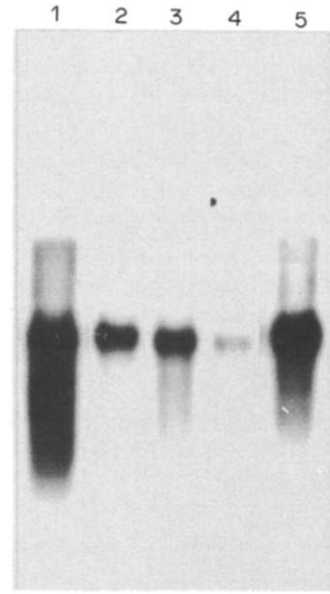

Fig. 5. Androgenic repression of the hepatic SMP-2 mRNA analyzed by Northern blotting. 1: adult female; 2: adult male; 3: ovariectomized female + vehicle; 4: ovariectomized female injected daily for 2 weeks with 5α-dihydrotestosterone (50 μg/100 g body wt); 5: testicular feminized male.

SMP-2 is an androgen-repressible protein [11]. Dihydrotestosterone injection into ovariectomized rats causes a marked reduction in the steady state level of the hepatic SMP-2 mRNA (Fig. 5). Ovariectomy alone has only a slightly diminutive effect in the SMP-2 mRNA level. This result, along with the observation that androgen insensitive mutant male rats (testicular feminized males) exhibit a high level of SMP-2 in the liver, indicate that SMP-2 expression is under androgenic repression. That such hormonal repression operates primarily through a transcriptional mechanism is evident from the results of nuclear run off studies (Fig. 6). The rate of SMP-2 gene transcription is 5-fold higher in nuclei isolated from livers of vehicle-treated ovariectomized females than that of DHT-injected ovariectomized females. The SMP-2 system consequently offers an important model to study androgenic repression of a specific gene at the transcriptional level.

CALORIE RESTRICTION RETARDS AGE-DEPENDENT LOSS IN ANDROGEN SENSITIVITY OF THE LIVER

Since its early report by McCay and coworkers, the efficiency of the calorie restricted

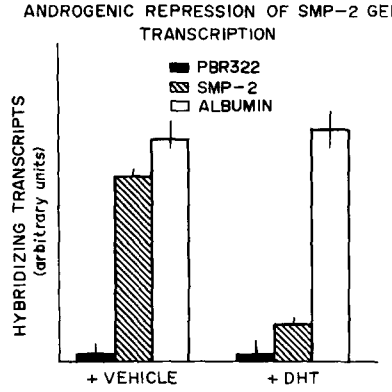

Fig. 6. Effect of DHT treatment on the SMP-2 gene transcription in the liver. Transcription run off assay using ovariectomized female rat liver nuclei isolated from the vehicle-treated control animal, and DHT-injected (50 μg/100 g body wt, one daily injection for 2 weeks) animal. The rate of transcription of the albumin gene did not vary in the two groups of animals. α-^{32}PUTP incorporation is >90% sensitive to α-amanitin (1 μg/ml). Each experimental point includes data from 4 individual animals.

diet in prolonging mean and maximum life span of rodents and other animals has been well-documented in many laboratories [19, 20]. The increased longevity is not due to the delay in growth and development, since food restriction initiated at an adult age is as effective as that begun at weaning [21]. Age-dependent dysfunctions in many physiological processes, as well as many age-associated diseases, can also be effectively postponed by this dietary manipulation [20]. It was of interest to explore therefore, what effect calorie restriction will have on the loss of androgen responsiveness of the liver during aging [22].

The Western blot analysis of Fig. 7 shows that, although male rats fed *ad libitum* (AL) fail to show any detectable level of CAB in the liver by 20 months of age, calorie restriction enables the liver to maintain the synthesis of CAB in rats as old as 27 months. Furthermore, the sustained androgen sensitivity of the liver in senescent male rats is accompanied by delayed age-dependent repression of the α_{2u}-globulin mRNA and derepression of the SMP-2 mRNA (Fig. 8). Quantitative analysis shows that for animals fed *ad libitum* the α_{2u}-globulin mRNA in 27-month-old rats is only 5% of that in 6-month-old animals. On the other hand, in animals maintained under a 40% reduction in food consumption since 42 days of age, 55% of the steady-state level of the α_{2u}-globulin mRNA is maintained at 27 months as compared to the 6-month level [22]. Quantitatively, this is equivalent to about 9 months of delay in the progression of the aging process within the period of 27 months. A decreased level of the androgen repressible SMP-2 mRNA in calorie restricted animals provides a strong supportive evidence for the molecular nature of the mechanism of extended longevity afforded by calorie restriction.

CONCLUSIONS

We have utilized the coordinate induction and repression of two androgen-responsive marker genes, i.e. α_{2u}-globulin and SMP-2, to study the molecular basis of age-dependent alterations in androgen sensitivity and gene expression of the hepatic tissue in the male rat. Transition from androgen resistance to the responsive state of the liver at maturation parallels pubertal appearance of a cytoplasmic androgen binding (CAB) protein in the rat liver. More recently, we have obtained evidence for the presence of the androgen receptor mRNA in the liver only during its hormone-responsive state. Hepatic androgen resistance during prepuberty and senescence is associated with loss of both the androgen receptor mRNA and the cytoplasmic androgen binding activity. The molecular relationship between CAB and androgen receptor is presently unclear. Preliminary observations suggest that pubertal appearance of the hepatic androgen sensitivity, CAB and androgen receptor mRNA, and androgenic induction of α_{2u}-globulin, coincide with the appearance of a male-specific *trans*-acting factor for the α_{2u}-globulin gene. Thus, the sensitivity of the hepatic tissue towards androgen is modulated through multiple regulatory processes involving developmental, hormonal and

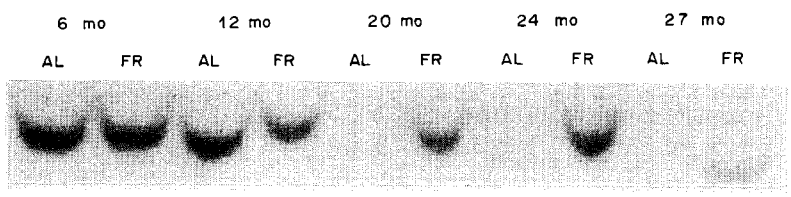

Fig. 7. The hepatic levels of immunoreactive CAB in *ad libitum* (AL) fed and food restricted (FR) male rats of increasingly advanced age.

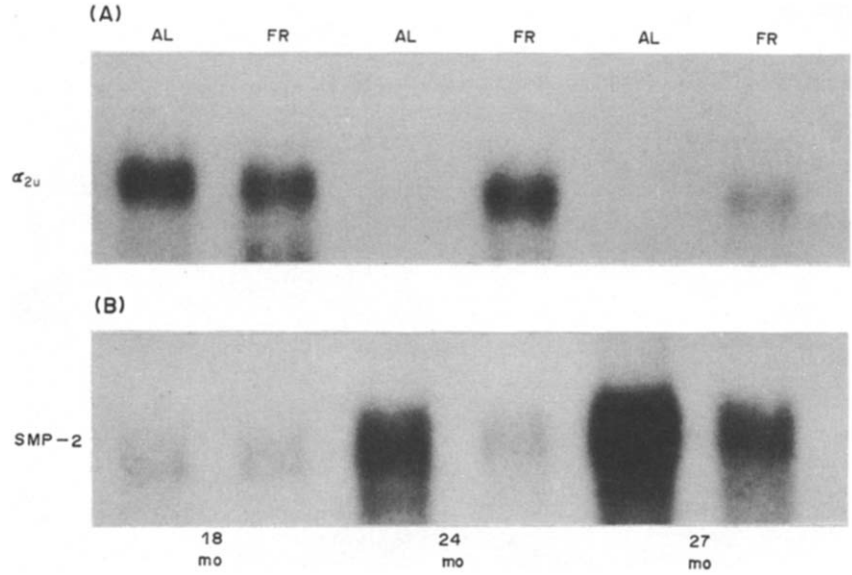

Fig. 8. The steady-state level of α_{2u}-globulin and SMP-2 mRNAs in *ad libitum* fed (AL) and calorie restricted (FR) rats during aging. The picture shows typical Northern blots of total hepatic mRNAs probed with either α_{2u}-globulin (A) or SMP-2 (B) cDNA. Equal amount of RNAs (20 μg) were applied to each lane of the gel. (The constitutive expression of the β-actin gene did not change with either age or dietary restriction.)

nutritional factors. Most of these regulatory events are reversed during senescence. However, the results of our nutritional studies show that the hormone insensitivity of the liver at old age toward the sex steroid androgen can be effectively delayed under a controlled calorie intake by the animal. Successful reversal of the hepatic andropause as well as many other age-related physiological dysfunctions in laboratory animals provides exciting possibilities for geroendocrinology.

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