Use of a cloned double stranded cDNA coding for a major androgen dependent protein in rat seminal vesicle secretion: the effect of testosterone in gene expression

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ABSTRACT

The abundant class of poly(A+)RNA [poly(A+)RNA\textsuperscript{115}] from rat seminal vesicle was used to synthesize ds-cDNA\textsuperscript{115}. The ds-cDNA\textsuperscript{115} was inserted and cloned into the Pst I site of pBR-322 using E. coli RR1 as host. Colony filter hybridization and restriction mapping was used to demonstrate that a 620 NTP long insert in a plasmid clone (pSV2) represents the almost full length structural gene coding for a precursor to the seminal vesicle secretion protein IV (SVS IV). The entire insert was sequenced and the coding region was matched with the known amino acid sequence. Most of the signal peptide sequence was derived from the DNA sequence. The insert in pSV2 was labelled and used to study the effect of testosterone on the accumulation of mRNA SVS IV. Administration of testosterone to castrated rats resulted in the induction of mRNA SVS IV from a few molecules per cell to levels of over 100,000 after 96 h of hormone treatment.

INTRODUCTION

In rat seminal vesicle there is a small set of polypeptides representing 30-50% of the proteins synthesized by the vesicle and the synthesis of these proteins is controlled by androgens (1,2,3,4). The abundant class of poly(A+)RNA coding for these polypeptides has been isolated and characterized (5). This report describes the cloning of double stranded cDNA (ds-cDNA\textsuperscript{115}) made to the abundant class of poly(A+)RNA [poly(A+)RNA\textsuperscript{115}]. Several were identified as having cDNA inserts coding for the abundant seminal vesicle polypeptides. One clone (pSV2) was shown to contain the entire coding sequence for the seminal vesicle secretion protein IV (SVS IV) by direct DNA sequencing. This cDNA insert (cDNA SVS IV) was then used to study the effect of testosterone on the accumulation of the mRNA from the SVS IV gene. This report describes these results.

MATERIALS AND METHODS

Preparation of seminal vesicle total poly(A+)RNA, isolation and characterization of poly(A+)RNA\textsuperscript{115} synthesis of single stranded cDNA and agarose gel

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electrophoresis have been described in detail (5).

Synthesis of ds-cDNA

Double stranded cDNA was synthesized mainly as previously described (6). The reaction contained 10 mM MgCl₂, 500 μM of TTP, dGTP and dATP, 100 μM dCTP, 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10-30 μg/ml of [²H]-cDNA, 50-100 μCi [α-³²P] dCTP (New England Nuclear) and 200 units/ml of AMV reverse transcriptase (from J. W. Beard Life Sciences, St. Petersburg, Fl.). The yield of ds-cDNA₁₁ was 0.10-0.15 moles of ds-cDNA per mole of template RNA. After treatment with Sl-nuclease (Bethesda Res. Lab.), 0.2 pg ds-cDNA₁₁ was tailed with ³H-dCTP by 10 units of terminal deoxynucleotide transferase (P-LBiochemicals) in the presence of 1 mM CoCl₂ (7).

Construction of recombinant plasmids

Plasmid pBR-322 was linearized with Pst I. The linearized pBR-322 was tailed with ³H-dGTP as mentioned above for ds-cDNA₁₁. Recombinant plasmids were formed by annealing 40 ng of tailed ds-cDNA₁₁ to equimolar amounts of tailed pBR-322 at 68°C for 1h. The reaction volume was 25 μl and the reaction buffer contained 0.1 M NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.4). The sample was left in the waterbath over night to slowly cool down and used for the transformation the following morning.

Transformation of E. coli RR1 and identification of clones

The transformation of E. coli RR1 was performed according to Stein et al. (8). Tet⁷/Amp⁵ colonies were screened by colony filter hybridization mainly as described by Grünstein-Hogness (9) with a ³²P-cDNA probe to enriched mRNA IV [prepared by sucrose gradient centrifugation(5)]. The DNA from clones showing a strong hybridization signal to the probe was isolated by growing the corresponding colonies in liquid culture to an OD₅₅₀ = 0.35 and preparing a cleared lysate (10) after amplifying overnight in the presence of 50 μg/ml chloramphenicol. The plasmid DNA was recovered by banding in a CsCl gradient, using propidium iodide (Calbiochem) (21) and passed through a CL-Sepherose 4B column equilibrated with 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 2 mM EDTA to remove any contaminating small RNA.

Restriction enzyme analysis

Restriction endonuclease enzymes were purchased from Bethesda Research Lab or New England Biolabs and used according to the manufacturer. Restriction fragments were analyzed on 6.5% polyacrylamide gels or 2.5% agarose gels (5) and bands were visualized by ethidium bromide staining (5 μg/ml) The DNA fragments were then transferred to a nitrocellulose paper according to Southern (11).
DNA sequencing

DNA sequencing was performed according to Maxam and Gilbert (12). The recombinant DNA experiments were performed in a P2 facility according to NIH guidelines.

Recovery of DNA insert from plasmid

The plasmid DNA (300-500 µg) was digested with Pst I (1 unit/µg DNA) overnight at 37°C and the sample was then electrophoresed in a 2% agarose gel as described (5). After staining with EtBr the insert could be identified. The gel region containing the insert was extracted with 100 mM NaCl-1 mM EDTA-50 mM Tris-HCl (pH 7.8)-0.2% SDS for three days at room temperature. The agarose was removed by centrifugation for 2 h at 40 K in a SW50 rotor and the supernatant was extracted twice with an equal volume of phenol-chloroform. The aqueous phase was made 0.2 M with trace and precipitated with ethanol. The precipitate was washed with 70% ethanol and dissolved in 20 mM NaCl.

Testosterone induction of mRNA SVS IV

Rats castrated for 5 weeks were injected twice daily with 1 µg testosterone propionate. At different time intervals rats in groups of ten were killed by decapitation and the seminal vesicles were removed. Total cellular RNA was isolated as described (15). The amount of mRNA IV in the total RNA population was determined by hybridization to the nick-translated (22) cDNA IV clone (spec. act. = 2x10⁹ cpm/µg). A kit from BRL was used for the nick-translation. Because of the high spec. act. of the ^32P-probe the self-renaturation of the probe during the hybridization to the total RNA could be limited to 7-8%. The hybridizations were performed as described in detail elsewhere (16). The kinetic standard was the nick-translated probe hybridized to poly(A⁺)₁₁S and we assumed mRNA SVS IV represents 50% of the mRNA in poly(A⁺)₁₁S (5).

RESULTS

Total poly(A+)RNA was isolated from rat seminal vesicles and the highly abundant class of poly(A+)RNA [poly(A+)RNA₁₁S] was recovered from a 5-20% sucrose gradient in 1% SDS (5). Agarose gel electrophoresis and in vitro translation of poly(A+)RNA₁₁S have shown that it contains at least two major poly(A+)RNA's, 620 and 540 NT long respectively, coding most likely for precursors to SVS protein IV and SVS protein V. The poly(A+)RNA₁₁S was used as template to synthesize ds-cDNA₁₁S in two consecutive reactions with AMV reverse transcriptase. The synthesis of ds-cDNA₁₁S was monitored by labelling
the first strand with $^3$H-dCTP and the second strand with $^{32}$P-dCTP. The $^{32}$P-ds cDNA$_{11S}$ was restricted with various restriction endonuclease enzymes and analyzed on 2.5% agarose gels. The results show that the undigested ds-cDNA$_{11S}$ consists of two major sequences, 600 and 520 NTP long respectively, with the 600 NTP sequence in preponderance (data not shown). These molecular weights correspond very well with those of the two abundant poly(A$^+$)RNA sequences present in poly(A$^+$)RNA$_{11S}$ and indicate that the synthesized ds-cDNA is of full length.

Construction of cDNA clones

The ds-cDNA$_{11S}$ was inserted into the Pst 1 site of pBR-322 (Ter$^r$/Amp$^r$) and used to transform E. coli RR1. By using the Pst 1 site the bacteria carrying the recombinant plasmids will be Tet$^r$/Amp$^s$ and subsequent screening for Ter$^r$/Amp$^s$ colonies will indicate where an inserted sequence might be present. Seventy-five percent of the Ter$^r$ colonies were sensitive to ampicillin. Colony filter hybridization was performed with the Tet$^r$/Amp$^s$ colonies (fig. 1A) (9). The $^{32}$P-cDNA probe ($^{32}$P-cDNA$_{IV}$) used for the hybridization was made to poly(A$^+$)RNA from a fraction from the faster sedimenting portion of the poly(A$^+$)RNA$_{11S}$ peak. This material was significantly enriched for sequences coding for a precursor to protein IV (5). The plasmid DNA from three colonies with strong hybridization signals (fig. 1A) was isolated and the size of the insert determined after restriction with Pst I (fig. 1B). It is known from Pst I digestion of $^{32}$P-ds-CDNA$_{11S}$ that at least one of the major ds-cDNA$_{11S}$ sequences do not contain any Pst 1 sites (data not shown). Furthermore, by using the G-C tailing method for the construction of the recombinant plasmids, the host will generate a Pst 1 site on both ends of the insert. The results show that two recombinant plasmids, pSV2 and pSV35 contain inserts 620 NTP long. The insert size together with the result from the colony filter hybridization indicate that pSV2 and pSV35 contain most likely inserts representing close to full length structural gene coding for SVS protein IV (gene$_{IV}$). One of the clones, pSV2 was chosen for the following studies.

Restriction analysis of the pSV2 insert

The Hind III and Pvu II fragmentation pattern (fig. 3) also agree with the results from the restriction map of $^{32}$P-ds-CDNA$_{11S}$ (data not shown). In order to facilitate the sequencing and the ultimate identification of SVS IV structural sequences a series of restriction enzymes was used to generate a map of the pSV2-insert. The 620 NTP long insert was recovered from a preparative agarose gel as described (14) after digesting the plasmid with Pst I. The insert was restricted and the DNA fragments were analyzed
Fig. 1. (A) Colony filter hybridization to identify colonies carrying seminal vesicle DNA. The colonies were transferred and fixed to nitro cellulose filter, and the DNA was then hybridized to a $^{32}$P-cDNA probe prepared as described in the text. Clones 2, 5 and 35 were chosen for further analysis. (B) Analysis of insert size on 2.5% agarose gels. Plasmid DNA was cleaved with Pst I and run on the gel. Lane 1: HhaI digest of φX-174 DNA as standard, Lane 2: pSV 35, Lane 3: pSV5, Lane 4: pSV2.

on 6.5% polyacrylamide gels. A typical gel is shown in fig 3. The stained pattern in fig. 2A and a Southern blot of the same gel in 2B is presented. The transferred DNA fragments were hybridized to $^{32}$P-cDNA made to poly(A$^+$)-RNA$_{115}$. The stained pattern matches the pattern from the autoradiogram indicating that the cloned ds-cDNA represents a sequence from the poly(A$^+$)-RNA$_{115}$ population. The Hind III and Pvu II fragmentation pattern also matches very well the result from the restriction of $^{32}$P-ds-cDNA$_{115}$. The position of a given restriction site was determined by performing a double
Fig. 2. Electrophoresis of restriction fragments of the insert from pSV2. A Stained 6.5% polyacrylamide-gel run as mentioned in Materials and Methods. Lane 1: Pvu II, Lane 2: Pvu II/Hpa II, Lane 3: Hpa II, Lane 4: Hae III, Lane 5: Ava I/Pvu II. B. Southern blot of the same gel. The transferred DNA was hybridized to ^32P-cDNA11S. A Hae III digest of φx-174 DNA was used as standard.

digest with Hind III or Pvu II. This preliminary map was necessary for performing the DNA sequencing of the insert in pSV2. A more detailed restriction map could then be constructed by a computerized search for restriction
enzyme sites in the nucleotide sequence of the pSV2 insert. The result is shown in fig. 3.

**Nucleotide sequencing**

The insert from pSV2 was sequenced according to Maxam and Gilbert and the nucleotide sequence was compared to the known amino acid sequence of protein IV (13). The results (fig. 4) conclusively show that the 620 NTP insert in pSV2 contains the entire sequence capable of coding for protein IV, as well as the coding information for 18 out of 21 of the amino acids comprising the signal peptide. The insert in pSV2 also codes for the entire 3'-end of the structural gene for SVS IV. Furthermore, there is a 210 bp untranslated region and as in other structural genes the sequence AATAAA close to the 3'-end (27 bp). The sequencing experiment also reveals the 5'-3' orientation of the insert. The nucleotide sequence was thus matched to the amino acid sequence and confirms that pSV2 contains an almost full-length insert of DNA representing the structural gene for SVS IV.

**Testosterone induction of mRNA SVS IV**

The nick-translated cDNA IV probe coding for mRNAIV was used to determine the amount of mRNAIV induced after administration of testosterone for dif-
Fig. 4. Nucleotide sequence of cloned cDNA \textit{SVS IV} and the corresponding amino acid sequence. A few basepairs are missing from the 5'-end presumably due to cleavage of the hairpin loop in the preparation of double stranded cDNA for cloning. The complete amino acid sequence of the protein \textit{SVS IV} is from Pan, Y. C. et al. (1980) Int. J. Peptide Protein Res. 15 and the partial sequence for the signal peptide is from Ostrowski, M. C. et al. (1979) X1th International Congress of Biochemistry, p. 207 and from this DNA sequence.
ferent length's of time to rats castrated for five weeks. The $^{32}$P-labelled probe was hybridized to total cellular RNA as described in Material and Methods. The results are shown in fig. 5. The level of mRNA$_{IV}$ in seminal vesicles from castrated rats was found to be extremely low. High Crot values were necessary to detect mRNA$_{IV}$ and the hybridizations did not go to completion but well over 50%. Therefore, an estimation of Crot $\frac{1}{2}$ could still be performed since at Crot = $7 \times 10^2$, 60% of the $^{32}$P-probe hybridized. A several fold increase in the concentration of mRNA$_{IV}$ could be detected already after 4 h of treatment with testosterone. This sharp increase continued for another 90 h. After 96 h of administration of testosterone the concentration of mRNA$_{IV}$ is approaching the level found in normal rat seminal vesicles. The data is summarized in table I. The calculations are based on a DNA content of 8 pg/cell (1) (23).

DISCUSSION

The highly abundant class of poly(A$^+$)RNA [poly(A$^+$)RNA$_{115}$] from rat seminal

![Graph](image_url)

Fig. 5. Hybridization of nick-translated cDNA IV insert to total RNA from rat seminal vesicle isolated from rats castrated for five weeks (-○○○○-); five weeks castration and treated with testosterone for 4 h (-△-△-△-), 24 h (-○-○-○-), 60 h (-■-■-■-) and 96 h (-▲-▲-▲-), normal control (-●-●-●-).
TABLE I

Approximate no. of molecules of mRNA IV/rat seminal vesicle epithelial cell.

| Time after androgen | Crot ½ | □ Fraction of total RNA mRNA SVS IV | *RNA/DNA ratio | *Fraction epithelial cells (E) | □ mRNA SVS IV per E cell × 10⁴ | Approximate no. of molecules |
|---------------------|--------|---------------------------------|----------------|-------------------------------|-----------------|-------------------------------|
| 5 weeks castrate    | 5 · 10² | 6 · 10⁻⁷                        | 0.70           | 0.4                           | 0.07            | 24                           |
| 4h                  | 1.5 · 10⁰ | 2 · 10⁻⁵                      | 0.70           | 0.4                           | 2.3             | 690                          |
| 24h                 | 1.6 · 10⁰ | 1.9 · 10⁻⁴                    | 1.4            | 0.4                           | 49              | 16,000                       |
| 60h                 | 5 · 10⁻¹  | 6 · 10⁻⁴                      | 2.6            | 0.65                          | 191             | 61,000                       |
| 96h                 | 3 · 10⁻¹  | 1.0 · 10⁻³                    | 2.8            | 0.65                          | 367             | 117,000                      |
| normal              | 1.8 · 10⁻¹ | 1.7 · 10⁻³                   | 3.4            | 0.70                          | 660             | 220,000                      |

\[ \text{Crot } \frac{1}{2} \text{ std.} \quad \text{Crot } \frac{1}{2} \text{ unknown} \]

\[ \text{Crot } \frac{1}{2} \text{ std } = 3 \cdot 10^{-4} \quad \text{(Determined from c-DNA SVS IV } - 11S \text{ mRNA hybrid)} \]

* From: Higgins, S.J., et. al. (1976) Biochem. J. 160, 43-48

Table I. Induction of mRNA IV in seminal vesicles from castrated rats treated with testosterone for various lengths of time.

vesicle was used as template to synthesize ds-cDNA₁₁S. The ds-cDNA₁₁S was inserted into pBR-322 and the recombinant plasmid was used to transform E. coli RR1. One clone (pSV2) containing a 620 NTP long insert was chosen for further analysis. The size of the insert is approximately the same as the size of the poly(A⁺)RNA sequence coding for a precursor to SVS IV (5). The size of mRNA IV together with DNA sequencing indicated that the insert is missing a few basepairs at the 5' end.

The insert size together with the result of the hybridization of insert fragments to \(^{32}P\)-cDNA probes and the sequencing data prove that the insert corresponds to an almost full-length structural gene coding for a precursor to SVS protein IV. It is well established that the synthesis of SVS protein IV is controlled by testosterone (4). In this report we have used the cloned structural gene to determine the concentration of mRNA IV after administration of testosterone to rats castrated for five weeks. The result (table I) show that testosterone can increase the amount of mRNA IV dramatically during a 96 h treatment with the hormone. The number of molecules/epithelial cell increases from approximately 25 to 100,000. The number of molecules found in normal seminal vesicles was approximately 200,000. The equivalent Cot ½ used
as a standard for the calculation of number of molecules of mRNA IV (table I) is based on the assumption that 50% of poly(A+)RNA\textsubscript{11S} represent mRNA IV. Based on previous hybridization studies (5) the mRNA IV content in poly(A+)RNA\textsubscript{11S} should be somewhere between 25 and 50%. If 25% represents the mRNA IV content in the poly(A+)RNA\textsubscript{11S} population, the number of mRNA IV molecules found in an average rat seminal vesicle epithelial cell would be 50% less, which in the case of the normal intact animal would correspond to about 100,000 mRNA IV molecules per epithelial cell. After inspection of poly(A+)RNA\textsubscript{11S} displayed in a denaturing agarose gel (5) we assumed that 50% of poly(A+)RNA\textsubscript{11S} is mRNA IV. However, even after taking the uncertainty of the mRNA IV content in the poly(A+)RNA\textsubscript{11S} into account it does not significantly alter the conclusions with respect to mRNA IV induction by the hormone testosterone presented above. The level and the kinetics of induction of mRNA\textsubscript{IV} in rat seminal vesicles is similar to the induction of abundant mRNA in rat ventral prostate (17) and the induction of ovalbumin mRNA in chick oviducts (18). The difference with respect to hormone influence, between seminal vesicle and the other two examples mentioned above seems to be in the turnover rate of abundant mRNA. The concentration of ovalbumin mRNA in estrogen stimulated chick oviducts (18), ventral prostate poly(A+)RNA from testosterone stimulated castrated rats (17) and the casein mRNA in lactating mammary glands (20) decrease rapidly after hormone withdrawal. Higgins et al. (19) have presented results that indicate only a three-fold decrease of the abundant mRNA sequences after seven days of testosterone withdrawal.

Since mRNA IV is a major component of the abundant class (5), and if a high level of mRNA IV is present in the seminal vesicle after one week of castration, this may imply that the ability of this system to destroy accumulated mRNA after hormone withdrawal may work considerably slower, as compared to other systems. The level of mRNA IV in seminal vesicles from rats castrated for one week through five weeks is presently being determined to confirm this hypothesis.

SVS protein IV has been described (1,2,3,4) and the amino acid sequence has been deduced (13). The biological function of protein IV is still unknown but considering that this protein represents 25-30% of the seminal vesicle protein-synthetic output an important role in reproduction seems very likely.

Presently we are isolating the genomic sequences homologous to pSV2 in order to define in detail the structure of this gene and surrounding sequences. The restriction map derived from the sequence data should prove helpful.
Comparison of future clones of genomic DNA sequences coding for SVS V with those coding for SVS IV should provide useful information on coordinate control of a small family of androgen regulatable genes.

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