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Development 121, 1205-1216 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The cAMP Response Element Binding protein is involved in hydra regeneration

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SUMMARY

Hydra provides an interesting developmental model system where pattern formation processes are easily accessible to experimentation during regeneration. Previous studies have shown that the neuropeptide head activator affects cellular growth and head-specific cellular differentiation during head regeneration and budding. In order to investigate the signal transduction pathway and the regulatory genes involved in these processes, we measured cAMP levels after head activator treatment and found that head activator leads to an increase in cAMP levels at concentrations where effects on nerve cell determination and differentiation are observed (10^{-11} to 10^{-9} M). Moreover, exposure of intact hydra to a permeable form of cAMP stimulates nerve-cell differentiation and thus mimicks the effect of endogenous head activator. Band-shift assays were performed to detect changes in hydra nuclear protein binding activity during regeneration or after head activator treatment. We found that the cAMP response element (CRE) promotes a specific and strong DNA-binding activity which is dramatically enhanced and modified during early regeneration or after HA treatment. We also identified a surprisingly highly conserved hydra gene encoding the cAMP Response Element Binding protein, which is involved in this CRE-binding activity. Initiation of regeneration upon wounding provokes an endogenous release of HA which leads to the final differentiation of determined nerve cells. We propose that the nerve-cell differentiation observed within the first 4-8 hours of regeneration relies on the agonist effect of head activator on the cAMP pathway, which would in turn modulate the CRE-binding activity of the hydra CREB protein and thus regulate the transcriptional activity of genes involved in regeneration processes.

Keys words: hydra, regeneration, nerve cell differentiation, head activator neuropeptide, cAMP pathway, CREB transcription factor

INTRODUCTION

Hydra is a freshwater coelenterate that provides an interesting model for developmental biological studies since it is one of the most primitive organisms displaying a head to foot polarity, resulting from a continuous process of head- and foot-specific differentiations leading to pattern formation. This differentiation involves few cell types with short differentiation pathways. It is initiated in the undifferentiated body column and is finalised in the extremities of the animal. Thus, the central part of the animal, called the gastric column, contains predominantly undetermined, undifferentiated cells. In contrast, the two ends of the animal, the tentacles and the foot are mostly made up of terminally differentiated cells or cells committed to that process. The two cell layers, ectoderm and endoderm, are separated by an extracellular collageneous structure, the mesoglea. These two layers consist predominantly of epithelio-muscular cells and of interstitial cells which constitute the stem cells for nerve cells, gland cells, nematocytes and for gametes in the sexual cycle. Under standard laboratory culture conditions most hydra species reproduce asexually by budding and have a very high regeneration and reaggregation potential.

Regeneration is of particular interest as it is a morphallactic process which involves no growth at least within the first hours after cutting (Park et al., 1970; Holstein et al., 1991). The remaining gastric tissue undergoes determination and differentiation leading to the re-establishment of positional values (Wolpert et al., 1974). The injury effect has been shown to be a prerequisite to regeneration and, as stimulation of nerve cell differentiation is an early event following wounding of the animal (Holstein et al., 1986; Hoffmeister and Schaller, 1987), nerve-cell differentiation is believed to play a key role in regenerative processes.

Two types of signals control the ordered spatial and temporal patterns of differentiation resulting in morphogenesis: positive signals induce specific, local differentiation events, and negative signals inhibit the spread of such inductions to larger areas (reviewed by Schaller et al., 1989). Head-specific growth and differentiation processes are initiated by head activator (HA) and inhibited by head inhibitor (HI). Foot-specific development depends on the presence of foot activator

(FA) and is inhibited by foot inhibitor (FI). Both activators are neuropeptides whose biological effects can most easily be measured as acceleration of head or foot regeneration; similarly, inhibitors which were copurified with nerve cells, can retard or completely inhibit regeneration (Schaller et al., 1979).

HA, which was isolated several years ago (Schaller and Bodenmüller, 1981), has been shown to be distributed in its granule-stored form as a head to foot gradient along the animal (Schaller and Gierer, 1973). At low concentration, HA acts as a growth factor by forcing undifferentiated cells to go through G_2 and divide; at higher concentrations, HA is a signal for nerve-cell determination and differentiation and for head-specific determination and differentiation of epithelial cells (Schaller et al., 1989). Thus, as in hydra nerve cells secrete substances controlling cellular growth and differentiation, one can postulate that nerve differentiation and pattern formation are tightly linked in normal hydra development.

All these processes probably require protein synthesis and hence should elicit changes at the level of gene expression. Therefore, to elucidate at the molecular level the visible events of regeneration, we investigated the signal transduction pathway of head activator and we searched for hydra nuclear proteins exhibiting variations in their DNA-binding activity during regeneration and after HA exposure. Our results show that cAMP might be a second messenger targeted by HA to achieve its cell-determination and -differentiation effects through the cAMP response element on responsive genes. The nuclear proteins involved in the CRE-binding complex show a dramatic modification of their DNA-binding activity during regeneration or after HA treatment. A hydra cAMP response element binding protein (CREB) related gene was isolated and its product participates in this CRE-binding complex as evidenced by the super-shift observed in the presence of a specific hydra CREB antibody.

MATERIAL AND METHODS

Culture of animals and regeneration procedure

Hydra were cultured as described by Hoffmeister (1991). After 24 hours starvation 1000 animals per time point were cut horizontally in the middle of their gastric region and head and foot halves were separately collected and left for regeneration in Petri dishes.

Head activator

HA is an undecapeptide of the sequence pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe. The HA bipeptide, C_8-HA[2] (Neubauer et al., 1990) was used in all experiments except those shown in Fig. 2B,C where glutaraldehyde-coupled HA[2] was taken (Schaller et al., 1990).

cAMP assays

For each condition, 20 budless animals were collected and transferred to vials containing 4.5 ml of hydra medium. They received then 0.5 ml of hydra medium containing 2 mM IBMX solubilized in 100% DMSO and head activator (HA[2]) freshly diluted at concentrations ranging from 10^{-7} to 10^{-12} M. Treatment was performed at room temperature for 10 and 30 minutes and stopped by briefly vortexing the animals, aspirating the medium and replacing it with 1.5 ml of cold 100% ethanol. Tubes were left on ice for 5-10 minutes before the animals were sonicated and the homogenate spun for 5 minutes at 2500 ***g***. The supernatant was then lyophilised and resuspended in 200 μl of 50 mM acetate buffer, pH 5.8, 0.01% sodium azide. Cyclic AMP was assayed using a radioimmunoassay kit (RP509, Amersham) and results were calculated as the ratio of fmol of cAMP per μg of protein (Bradford, 1976).

Nuclear extracts and band-shift assays

Nuclear extracts were prepared as described by Dignam et al. (1983) with the following modifications**.** 1000 unfed animals were washed twice in hydra medium and once briefly in buffer H (10 mM Hepes, pH 7.9, 2 mM $MgCl_2$, 5 mM KCl, 0.5 mM spermidine, 0.15 mM spermine). They were then douced in 10 ml of buffer H (30 strokes) and 2.1 ml of a 2 M sucrose solution were slowly added during the next 10 strokes. All manipulations were performed on ice in buffers containing 1 mM DTT, 1 mM benzamidine, 0.5 mM β-mercaptoethanol, 0.5 mM PMSF added just prior to use, as well as a mix of protease inhibitors (100×: 200 μg/ml pepstatin, 50 μg/ml leupeptin, 100 μg/ml aprotinin, 400 μg/ml bestatin, used 1× in hypotonic and elution buffers, 0.1× in binding buffer). After centrifugation (1500 ***g***, for 10 minutes) the pellet was washed twice in 8 ml of buffer S (buffer H containing 0.3 M sucrose) and resuspended in 5 volumes of buffer E (10% glycerol, 400 mM NaCl, 10 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine). After gentle stirring for 45 minutes, the eluate was spun for 20 minutes at 18000 ***g*** and the supernatant precipitated by addition of ammonium sulphate (0.40 g/ml). The precipitate was left on ice for 30 minutes and then spun for 30 minutes at 18000 ***g***. The pelleted proteins were dissolved in 500 μl of buffer B (10% glycerol, 12 mM Hepes, pH 7.9, 4 mM Tris-HCl, pH 8, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1mM EDTA, 1.6 mM spermidine) and dialyzed against the same buffer. After a short centrifugation, the supernatant was aliquoted, frozen in liquid nitrogen and stored at −70°C. The protein concentration (Bradford, 1976) was usually around 2 mg/ml. Band-shift assays were performed by incubating for 15 minutes at room temperature 10 μg of nuclear extract in 15 μl of buffer B containing 5 fmoles (10000 c.p.m.) of an end-labelled oligonucleotide, 0.266 mg/ml BSA and 0.2 mg/ml ssDNA. Each sample was then loaded onto a 4% polyacrylamide gel and electrophoresed at 4°C and 250 volts for 90 minutes with recirculated buffer (0.5× TBE).

Cloning strategy, cDNA library screening and PCR sequencing

A hydra *CREB* cDNA clone was initially isolated from an oligo-dT primed λgt10 cDNA library of the multiheaded mutant *Chlorohydra viridissima* screened under the conditions described by Schummer et al. (1992) with an end-labelled 48 guessmer (AAA C/AGI GAA GTI C/AGI C/TTI ATG AAA AAT C/AGI GAA GCI GCI C/AGI GAA TGT) which encodes part of the basic domain of the rat CREB protein (KREVRLMKNREAAREC) (Gonzalez et al., 1989) and follows the hydra codon usage (Galliot and Schummer, 1993). Only partial clones representing one type of cDNA (*cv.CREBα*) were purified from this library; the longest (715 bp) contained an ORF ending in the basic region without stop codon. By use of inverted PCR (Ochman et al., 1990) on *Hin*dIII digested *cv.* genomic DNA, 1.8 kb of additional flanking sequences of *cv.CREB α* were obtained, which extended the initial ORF by a full leucine zipper domain preceding a stop codon. *cv.CREB α* cDNA was used as a probe for genomic Southern analysis under conditions described by Schummer et al. (1992) and for screening an oligo-dT primed λgt10 *Hydra vulgaris* cDNA library from which two types of cDNAs were isolated: *hv.CREBα*, partial, 250 bp long and *hv.CREBβ*, 1429 bp long.

Expression of the hydra CREB protein in *E. coli*, production of polyclonal antibodies and western analysis

A *cv.CREBα* cDNA was cloned into the IPTG-inducible expression vector pQE-30 (Qiagen) to produce the plasmid pQE-CREB165cv. The resulting protein includes 6 histidine residues at the N-terminal end and covers 165 amino acids from position 43 to position 207 of the complete 249 aa long cv.CREBα protein. The *E. coli* strain,

SG13009[pREP4] was transformed with pQE-CREB165cv and induced to express the recombinant protein by addition of 1.5 mM IPTG to a growing culture at an OD_{600} of 0.8. Recombinant CREB protein was purified by nickel chelate affinity chromatography (Hochuli et al., 1988) under denaturing conditions, using 6 M guanidine hydrochloride as a solubilizing agent. Purified protein was concentrated by ultrafiltration (Centrisart I, SM 13229 E, Sartorius) according to the suppliers protocol. 100 μg of protein mixed with Freund's adjuvants were taken for each injection into rabbits. Boosts were given every month and sera were collected 10 days after each injection.

For western analysis, crude extracts were prepared from approximately 100 animals of each species. Animals were washed once in hydra medium and transferred to an Eppendorf tube where two volumes of S buffer (125 mM Tris pH 6.8, 1 mM $CaCl_2$, 1 mM PMSF) were added. Sonication was then performed on ice for 60 seconds and the homogenates were spun for 10 minutes at 13000 ***g*** in the cold. After centrifugation, the supernatants were aliquoted, frozen in liquid nitrogen and stored at −70°C. 20 μg of these extracts were diluted in 1× sample buffer (1% SDS, 100 mM Tris, pH 6.8, 0.4 mM EDTA, 8.5% glycerol, 0.05% bromophenol blue), electrophorezed on a 12% SDS-PAGE and electroblotted to nitrocellulose. The filter was colored with Ponceau-S, blocked for 1 hour in TBST (25 mM Tris, pH 8, 140 mM NaCl, 3 mM KCl, 0.1% Tween-20) containing 5% dry milk and then incubated overnight at 4°C in presence of the CREB antibody, pre-immune or immune serum collected after the third boost, diluted 1/1000 in 5 ml of TBST/1% BSA. The filter was washed 3 times for 10 minutes in TBST, incubated for 1 hour at RT in alkaline phosphatase coupled anti-rabbit IgG (Promega, diluted 1/10000), washed again 3 times for 10 minutes and revealed with Vectastain kit (Vector, SK 5300).

RESULTS

Cytoplasmic cAMP levels in hydra treated with HA and effects of cAMP on nerve-cell differentiation

As previous data indicated that cAMP might be involved during hydra morphogenesis (Wolpert et al., 1974; Hill and Lesh-Laurie, 1981; Holstein et al., 1986), we measured cAMP levels of whole hydra treated with HA for 10 and 30 minutes. A significant 2-fold increase was detected after 10 minutes when HA was used at concentrations ranging from 10^{-9} M to 10^{-11} M, but no significant change occurred at 10^{-13} M (Fig. 1A). This increase was no longer detectable after 30 minutes. Hydra treated with forskolin did not show any modification in their cAMP levels (data not shown).

One of the earliest visible effects of wounding or HA treatment on cell distribution is an increase in nerve-cell differentiation (Hoffmeister and Schaller, 1987). To assay whether cAMP can replace these stimulus, intact *Hydra vulgaris* were treated with a water-soluble, membrane-permeable form of cAMP (Sp-cAMP, Biolog). Since the number of epithelial cells remain essentially constant during the incubation, the nerve cell abundance was evaluated after 8 hours by measuring the percentage of nerve cells to epithelial cells (Fig. 1B). Upon cAMP treatment at 10 μM, this nerve cell ratio was, within 8 hours, significantly increased by two-fold (Fig. 1B), implying that cAMP exposure can mimick the fast nerve differentiation effect obtained upon HA treatment or wounding the animal. In a similar experiment, we showed that the HA determination effect can also be mimicked by the cAMP agonist (Fenger et al., 1994).

CRE-binding activity in hydra nuclear extracts

In mammalian cells several extracellular signals such as hormones, growth factors or neurotransmitters propagate their signal intracellularly by elevating the cytoplasmic level of cAMP, which in turn activates the protein kinase A (PKA) responsible for the phosphorylation of many proteins including nuclear transcription factors. These transcription factors regulate the expression of their target genes through the cAMP response element (CRE) which is present in promoters of a wide variety of cellular genes (Roesler et al., 1988). Both the CRE motif (TGACGT) and the transcription factors that bind to it are evolutionary conserved (Lin and Green, 1989a,b; Jones and Jones, 1989).

To detect whether HA, endogenously released upon wounding or exogenously applied to intact animals, targets the cAMP pathway also at the nuclear level, we used the band-shift assay to measure modulations in the CRE-binding activity of hydra nuclear proteins during regeneration or after HA treatment. As a first step, we searched for the hydra proteins exhibiting a CRE-binding activity. Nuclear extracts (Dignam et al., 1983) from hydra were assayed using two different CRE motifs carried on double-stranded oligonucleotides: ATFwt, which contains a consensus version designed according to Lin and Green (1989a) and CREwt, identical to the somatostatin CRE (Montminy et al., 1986). For each of these a mutated version, ATFmut and CREmut, was used as control (Fig. 2A). Three different species of hydra tested displayed a strong,

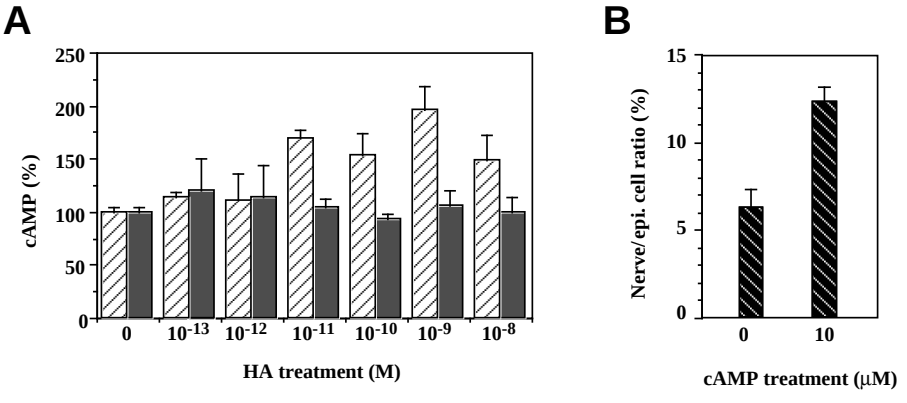

Fig. 1. (A) Effect of HA on cytoplasmic cAMP levels in hydra. Intact animals of *Hydra oligactis* were treated with increasing concentrations of HA. cAMP levels were measured after 10 (hatched bars) and 30 minutes (dark bars) in several independent experiments. After 10 minutes, HA at concentrations of 10^{-11} to 10^{-9} M induces an increase, usually 2- to 3-fold above the levels observed in control animals. Results from a representative experiment are depicted in this figure. (B) Effect of cAMP on nerve cell abundance. Intact *Hydra vulgaris* were treated with the cAMP agonist, Sp-cAMP (Biolog). The nerve-cell abundance was evaluated as the percentage of nerve cells to epithelial cells. This ratio was counted 8 hours after starting the cAMP treatment on hydra tissue dissociated into cells by maceration (David, 1973).

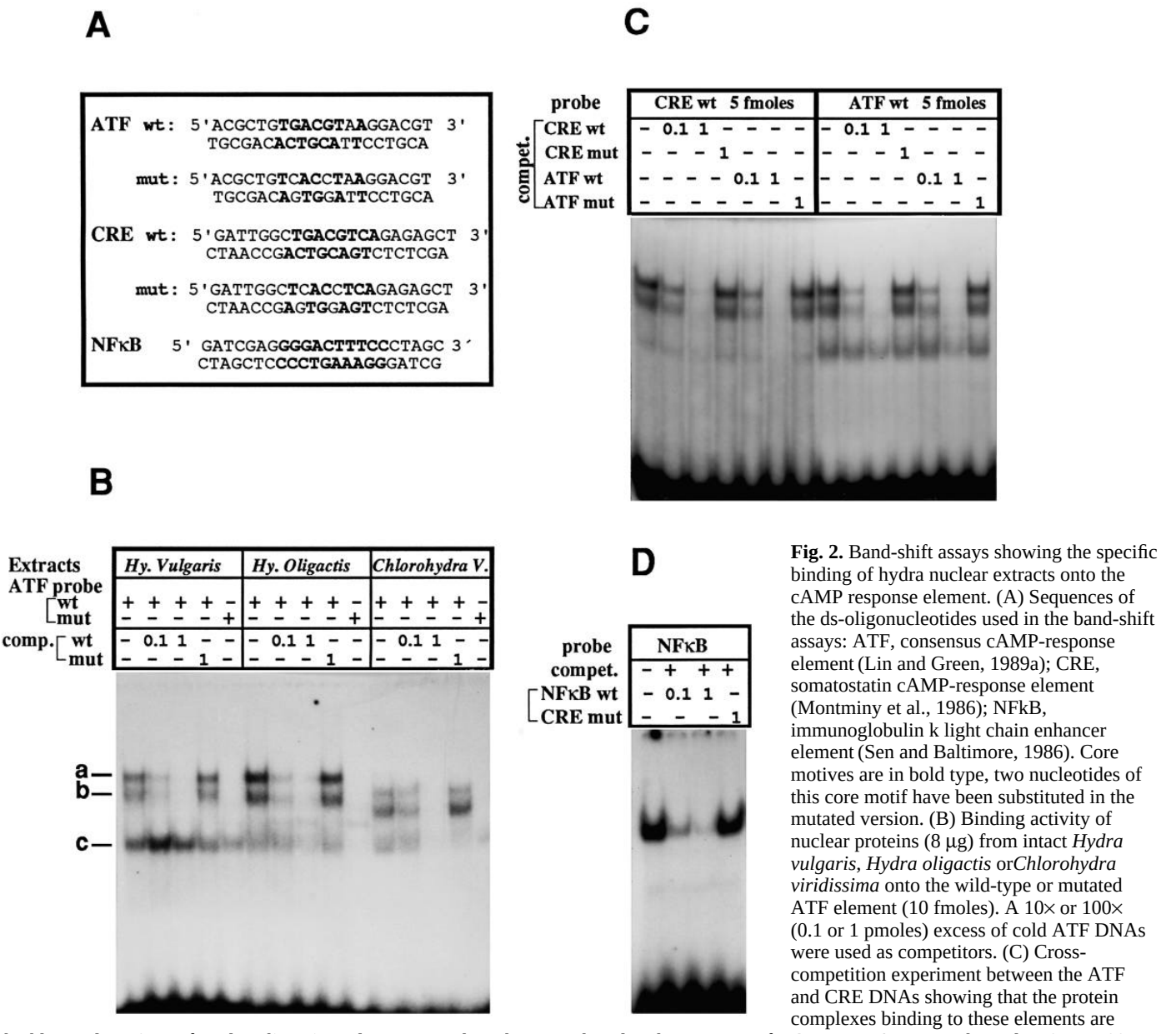

Fig. 2. Band-shift assays showing the specific binding of hydra nuclear extracts onto the cAMP response element. (A) Sequences of the ds-oligonucleotides used in the band-shift assays: ATF, consensus cAMP-response element (Lin and Green, 1989a); CRE, somatostatin cAMP-response element (Montminy et al., 1986); NFkB, immunoglobulin k light chain enhancer element (Sen and Baltimore, 1986). Core motives are in bold type, two nucleotides of this core motif have been substituted in the mutated version. (B) Binding activity of nuclear proteins (8 μg) from intact *Hydra vulgaris*, *Hydra oligactis* or*Chlorohydra viridissima* onto the wild-type or mutated ATF element (10 fmoles). A 10× or 100× (0.1 or 1 pmoles) excess of cold ATF DNAs were used as competitors. (C) Cross-competition experiment between the ATF and CRE DNAs showing that the protein complexes binding to these elements are highly similar. 10 μg of *Hydra oligactis* nuclear proteins have been incubated in the presence of a CREwt or ATFwt probe and a 20× or 200× (0.1 or 1 pmoles) excess of cold wild-type or mutated CRE or ATF DNAs were used as competitors. (D) The binding of hydra nuclear proteins onto the NFkB element is specific. 4 μg of *Hydra oligactis* nuclear proteins were incubated in the presence of a NFkB probe and a 10× or 100× (0.1 or 1 pmoles) excess of cold NFkB or unrelated (CREmut) DNAs were used as competitors.

specific binding activity to both ATFwt (Fig. 2B) and CREwt (Figs 2C, 3A, 7D). *Hydra vulgaris* and *Hydra oligactis* nuclear extracts showed three different major retarded bands, a, b, and c. As judged by the use of mutated ATF and by competition experiments (Fig. 2B), bands a and b were found to be highly specific whereas band c was less specific in *Hydra oligactis* and non-specific in *Hydra vulgaris*. The nuclear extracts of the multiheaded mutant of *Chlorohydra viridissima* exhibited a slightly different pattern with two major bands (Figs 2B, 7D). The protein complex binding to the CRE seems to be very similar to that binding to the ATF element as evidenced by the cross-competition experiment depicted in Fig. 2C: the retarded bands have the same size and can be competed with the same amount of cold ATF or CRE DNAs, only band c is more abundant when the ATF probe is used. Finally, the hydra CRE-binding complexes are different from that of human Hela cells as evidenced by their faster migration (not shown).

Changes in CRE-binding activity during regeneration and after HA treatment

Nuclear extracts from *Hydra oligactis* and *Hydra vulgaris* were prepared at various times during regeneration (Fig. 3A). In *Hydra vulgaris* head and foot are usually reconstituted after 2 to 3 days, while in *Hydra oligactis* head regeneration is normal, but foot regeneration is severely deficient (Hoffmeister, 1991). At the level of CRE-binding activity an initial dramatic change is detected during head regeneration of lower parts of *Hydra oligactis* (Fig. 3A): band *a* becomes much more intense and at

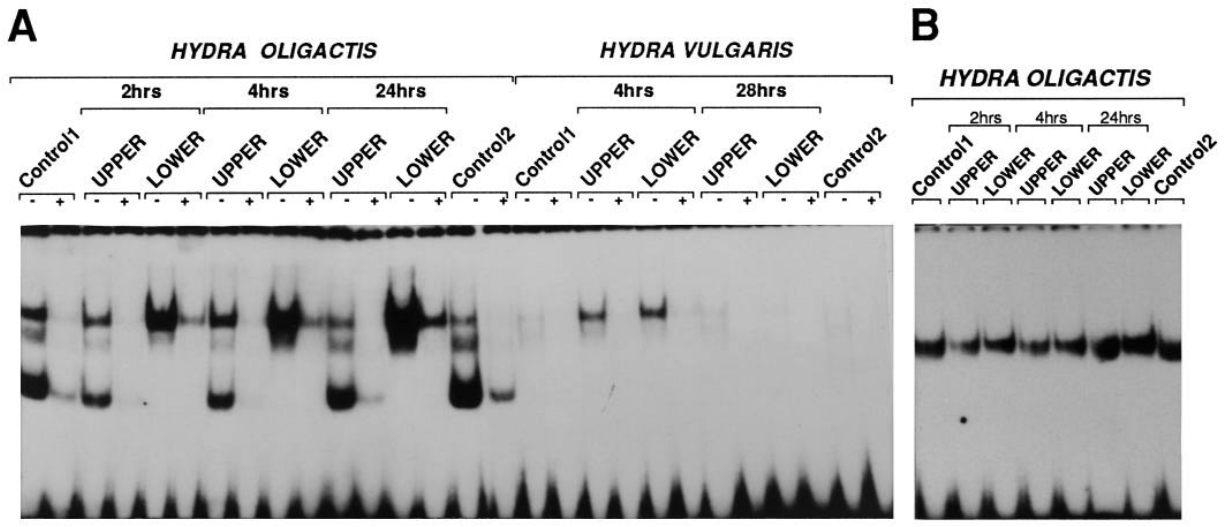

Fig. 3. Band-shifts assays showing the modifications of the CRE-binding activity during regeneration. Binding activity of nuclear proteins from regenerating *Hydra oligactis*, foot-regeneration deficient, (8 μg) and *Hydra vulgaris* which regenerate head and foot in 2 to 3 days (4 μg). Nuclear extracts were prepared at indicated times after cutting and incubated with CREwt (A) or NFkB (B) (only *Hydra oligactis*). UPPER, upper halves regenerating feet; LOWER, lower halves regenerating heads. + and – indicates the presence of cold CREwt competitor (100×).

the same time a decrease in the abundancy of the faster migrating complexes is observed. These changes are detectable as early as 2 hours after cutting, increasing in intensity from 2 to 24 hours. These modifications, also revealed by the ATF element (not shown), are not observed during foot regeneration of the upper parts. As foot regeneration is impaired in *Hydra oligactis*, we tested how the CRE-binding activity varies during regeneration of *Hydra vulgaris*. In this species an increase in the intensity of band *a* was found during both head and foot regeneration 4 hours after cutting (Fig. 3A). We therefore assume that at least the early changes are not head-regeneration specific, but concomittant to the regeneration processes *per se*. The NFkB DNA element (Sen and Baltimore, 1986), which is bound strongly and specifically by hydra nuclear proteins (Fig. 2D) was used to test the specificity of these modifications. The NFkB band-shift pattern was not drastically modified during regeneration of *Hydra oligactis* (Fig. 3B) suggesting that the CRE-binding proteins are specifically targetted upon this process.

In vertebrates the transcriptional transactivation function of the CREB protein results from phosphorylation at a very specific serine residue (Gonzalez and Montminy, 1989) controlled by the signal transduction cascade in which cAMP activates the protein kinase A (PKA). This posttranslational modification of CREB can modify its DNA-binding affinity (Weih et al., 1990; Nichols et al., 1992). To investigate whether this cAMP pathway also operates in hydra, intact *Hydra oligactis* were treated for 4 hours with cAMP agonists: forskolin (10 μM), dibutyryl cAMP (db cAMP, 1 mM) and 3-isobutyl-1-methylxanthine (IBMX, 2 mM). As shown in Fig. 4A, forskolin did not modify the ATF-binding activity, whereas db cAMP and IBMX did increase it. HA (10^{-9} M) tested in the same experiment also led to an increase of this binding activity.

The effect of HA was assayed in more detail with regard to the time course of action and concentration dependence. In the kinetic experiment performed on *Hydra oligactis* (Fig. 4B,C), HA induced modifications of the ATF/CRE-binding activity twice: during the first 15 minutes after HA application a fast and transient increase in binding was detected at 10^{-9} M and 10^{-11} M but not at 10^{-13} M. This effect was no longer present after 60 minutes. A second and stronger increase in bands a and b was observed after 2-4 hours of HA exposure, again at only the higher concentrations. This increase in CRE-binding activity was reduced after 12 hours and became negligible after 24 hours. These changes were similar when the somatostatin CRE or a consensus ATF were used (Fig. 4B,C). This fast increase observed in ATF/CRE-binding activity after HA treatment might be linked to the immediate rise in cAMP levels due to HA treatment. When the same extracts of HA-treated hydras were tested with the NFkB element (Fig. 4D) variations in the binding activities were also observed, although with somewhat different kinetics.

Cloning of the hydra *CREB* gene

In mammals, the CREB/ATF transcription factors, which regulate gene expression through CRE, belong to the bZIP family achieving DNA-binding through a basic region followed by a leucin zipper repeat required for dimerization (Brindle and Montminy, 1992). As a good candidate for the CRE-binding activity in hydra extracts, we cloned a hydra homologue of the mammalian *CREB* gene (Figs 5, 6). A guessmer oligonucleotide corresponding to the mammalian *CREB* basic region (Hoeffler et al., 1988; Gonzalez et al., 1989) was designed according to the hydra codon usage (Galliot and Schummer, 1993) and used in the initial screening of a *Chlorohydra viridissima* cDNA library. A first cDNA containing an open reading frame encoding a highly conserved DNA-binding domain was isolated and used for the subsequent screenings of *Chlorohydra viridissima (cv.)* and the *Hydra vulgaris (hv.)* cDNA libraries. In *Chlorohydra viridissima* a unique isoform was found, *cv.CREB* α, while two were detected in the *Hydra vulgaris* cDNA library, *hv.CREB*α and *hv.CREB*β differing only by the presence in *hv.CREB*α of a small additional exon, 51 bp long, also present in *cv.CREB*α at position 143 (Figs 5A and 6). All the cDNAs isolated cor-

responding to the *CREBα* isoform were partial at their 3′ end, stopping right after the DNA-binding region. From *cv.CREB*, additional genomic flanking sequences were obtained by use of the inverted-PCR strategy (Ochman et al., 1990). They were shown to contain an open reading frame encoding the basic region followed by a full leucine zipper domain that ends with a stop codon at the same position as the vertebrate *CREB* and *CREM* genes (Fig. 6). On *cv.* genomic southern this *CREB* gene was detected as unique (Fig. 5B). In *Hydra vulgaris*, the sequence of the *hv.CREBβ* isoform completely diverges in the carboxy-terminal part of the basic region and displays a stop codon before the leucine zipper domain, thus lacking the dimerization domain (Landschulz et al., 1988). Comparison between available *cv.CREBα* cDNA and genomic sequences revealed the presence of at least 4 introns, the most 3′ one (positions 1194-1268) being located within the basic region (Figs 5, 6). This donor splice site corresponds exactly to that detected in all isoforms of the mammalian *CREB* and *CREM* genes except in *CREBψ* (Ruppert et al., 1992; de Groot and Sassone-Corsi, 1993). The conserved position throughout evolution of this splicing event (Fig. 6) is remarkable, even though the length of this intron has dramatically changed from 74 bp in hydra to almost 20 kb in mouse (Ruppert et al., 1992).

The translation products of *cv.CREBα* and *hv.CREBβ* genes are 248 and 199 amino acids long respectively, shorter than those from the corresponding mammalian genes. The average similarity is about 35% (Fig. 6). The hydra CREB sequences display an extremely high degree of conservation in the bZIP region (85% identity over 72 residues). The sequencing of the 3′ flanking sequences of *cv.CREB* (1200 bp) did not show any additional bZIP motif as is the case for the *CREM* gene, which encodes two distinct bZIP domains. Transcriptional activation by CREB is achieved via phosphorylation by protein kinase A on a specific serine residue, the PKA site (Gonzalez and Montminy, 1989; Lee et al., 1990; Yamamoto et al., 1990). In the mammalian CREB/CREM proteins the 50 amino acids surrounding this site, the kinase inducible domain (KID or P-box) contain several consensus phosphorylation sites and the DLSSD motif which appear to cooperate with the PKA site to elicit transcriptional activation (Lee et al., 1990; Gonzalez et al., 1991). The N-terminal part (14 residues) of this domain is missing in the hydra CREB sequence, the region of the PKA site is identical over 16 amino acids and the adjacent DLSSD motif is present at the same location but modified (DLAGD). The C-terminal part of this domain, highly acidic and containing a consensus casein kinase II (CKII) phosphorylation site not critical for CREB function (Lee et al., 1990; Gonzalez et al., 1991), is less conserved in the hydra CREB sequence. Flanking the KID, two glutamine-rich domains were described in the CREB and CREM isoforms which behave as activators of transcription but are lacking in those acting as antagonists of cAMP-induced transcription (Foulkes et al., 1991; Molina et al., 1993). These two glutamine-rich domains, although reduced in length, are present in the hydra CREB protein sequence.

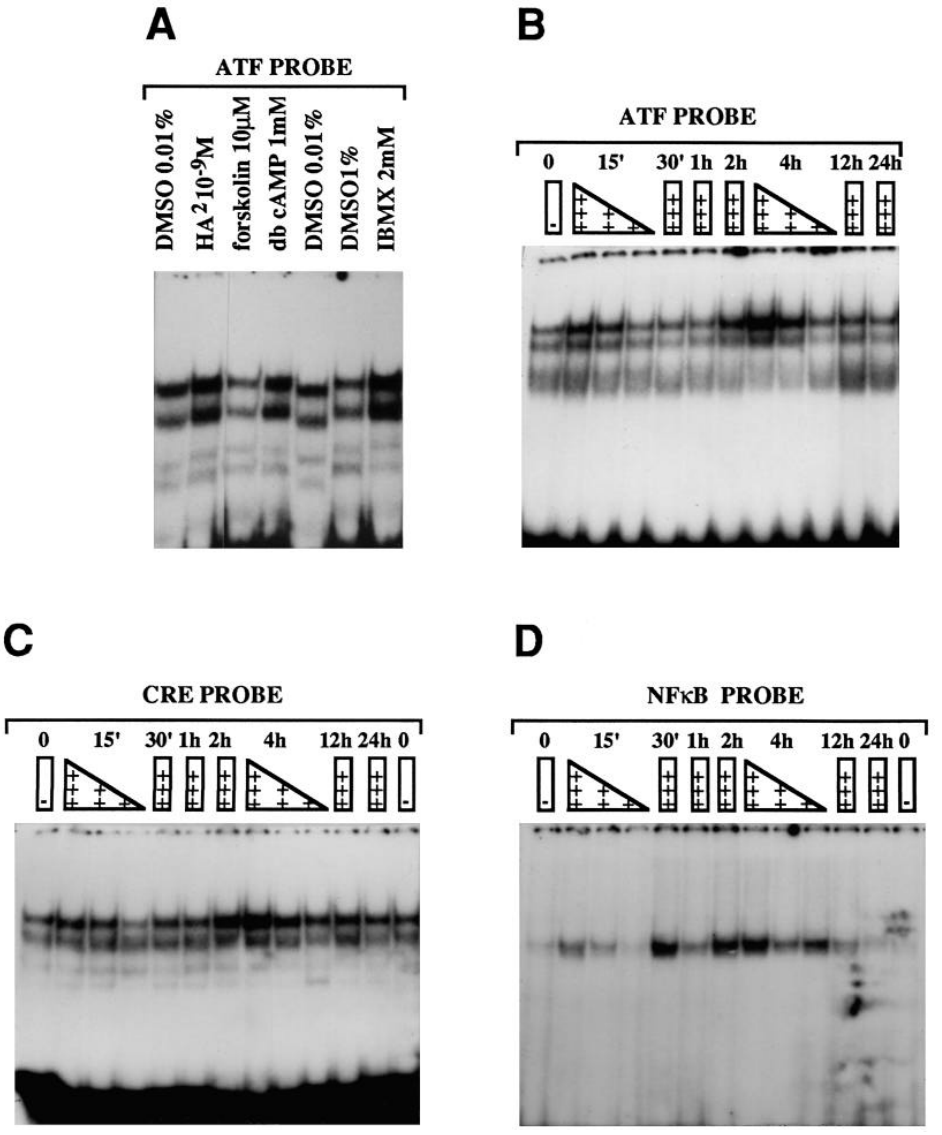

Fig. 4. Modification of ATF/CRE binding activity by treatment of *Hydra oligactis* with various cAMP agonists or HA. (A) Whole animals were treated for 4 hours with HA (1 nM), forskolin (agonist of adenyl cyclase, 10 μM), dbcAMP (permeable form of cAMP, 1 mM) in hydra medium containing 0.01% DMSO or with IBMX (inhibitor of cAMP phosphodiesterase, 2 mM) in hydra medium containing 1% DMSO. The binding of 15 μg of nuclear proteins was tested using the ATFwt element. (B-D) Band-shift assay with 15 μg of nuclear proteins from whole animals treated for 15 minutes to 24 hours with HA at three different concentrations: +++ (10^{-9} M), ++ (10^{-11} M), + (10^{-13} M), – (no HA). The binding was tested using the ATF (B), CRE (C) and NFkB (D) elements (sequences in Fig. 2A).

A

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cv.CREBα          CACAACTTGAGCATCCAAGAAAGCGTAACAAATAACTTGTTGAAGTTTATTTATTACCACGAGGGTAATAGAAATA......aagctttatgtttctacactattcttaacta   107
hv.CREBβ  CTCGTCG******C*************************************A*******C**T******************                                  83

cv.CREBα  agtcgcagcatgaatttattctgctcaccaaatgttatgccaaactgaactgttatttgagttttgcattgatcttttaattatattggctatttgataacattttgaggcagatcattt   227
cv.CREBα  ttcaaaagacttggatcttgttttaacccaagaaacagttttgctggaatgctgttgatctgagctgtaatgcttacagctcttgctgttggctttttaagtatttatttgctgaccatt   347

cv.CREBα  taaattaacattaagagttgtacttttcatttccattgattacttacaaaaatttgctttagGATTATGGAGTTGGCTCGCCATTCATTTCAGCAACCATTAAATGTTGCTCCTAGCTTA   467
                                                                    M  E  L  A  R  H  S  F  Q  Q  P  L  N  V  A  P  S  L     18
hv.CREBβ                                                          ***********A***AGA**********A**********************T***  140
                                                                    M  E  L  A  R  H  S  F  Q  Q  P  L  N  V  A  P  S  L     18

cv.CREBα  TCAAATGTTGTTAATAAAACATCTGTGGTGCTTCAACAACAGTCAGTAATCCAACCCAATCAGCACCAACTGCAGCACCAGCTGCAAACTATGCATGATGGGGGCATTGATGGGAAGAGA   587
          S  N  V  V  N  K  T  S  V  V  L  Q  Q  Q  S  V  I  Q  P  N  Q  H  Q  L  Q  H  Q  L  Q  T  M  H  D  G  G  I  D  G  K  R     58
hv.CREBβ  *****---***********G*****A**********************T***T*A********T*****A*****T**A*******A***************T*****A**A**A***  257
          S  N  -  V  N  K  T  S  V  V  L  Q  Q  Q  S  V  I  Q  S  N  Q  H  Q  L  Q  H  Q  L  Q  N  M  H  D  G  G  I  E  G  K  R     57

cv.CREBα  AGAGAAATACTAGCTAGAAGGCCTTCATACAGGCGTATACTTGATGATCTTGCAGGAGATGGACCTGTAAAAATGGAAAATTATGATGATACTGGAAGTAGTGGTGAATCCAGCCCAAAT   707
          R  E  I  L  A  R  R  P  S  Y  R  R  I  L  D  D  L  A  G  D  G  P  V  K  M  E  N  Y  D  D  T  G  S  S  G  E  S  S  P  N     98
hv.CREBβ  **********G********A**A*****T**A****************G**********T**A*********************C*****C********A**G**A**T******  377
          R  E  I  L  A  R  R  P  S  Y  R  R  I  L  D  D  L  A  G  D  G  P  V  K  M  E  N  Y  D  D  T  G  S  S  G  E  S  S  P  N     97

cv.CREBα  GGAAACAATGAAGAAGATATCAATGGAATAAATCAAAGTGTGAGCCATCAGGAAAAACAGTATCAAAGTATACACTTAAATGGAATTGTTTCATCTGTACAAGGAGGTGAAAATTCATTG   827
          G  N  N  E  E  D  I  N  G  I  N  Q  S  V  S  H  Q  E  K  Q  Y  Q  S  I  H  L  N  G  I  V  S  S  V  Q  G  G  E  N  S  L    138
hv.CREBβ  *****T**************T**C**T*******C**A*TCA*TT*****A********A*******A******T*******************A**T*****T**A*********A**  497
          G  N  N  E  E  D  I  N  G  I  N  P  N  S  I  H  Q  E  K  P  Y  Q  N  I  H  L  N  G  I  V  S  S  V  Q  G  G  E  N  S  M    137

cv.CREBα  AATCAGTTGCATGATTCGCAGCCAGGTGATAATCAATACATAATAACAACACAAGGACCAGACAATAAAATACAGgtagctctcatgtgttctttaatttaatttttttttttcttttttt   947
          N  Q  L  H  D  S  Q  P  G  D  N  Q  Y  I  I  T  T  Q  G  P  D  N  K  I  Q                                          163
hv.CREBβ  ********A********T***                                                                                              518
          N  Q  L  H  D  S  Q                                                                                                144
hv.CREBα   *******A********T*****C**C*****C***************G**********T***************
             Q  L  H  D  S  Q  P  G  D  N  Q  Y  I  I  T  A  Q  G  P  D  N  K  I  Q

cv.CREBα  ctttttttagtctttaattgaggcattaaatcttttgatcaatccatcacagttctaataatttttttaaatttagtttaacttttttttttttttttttgatttttgtctgatttt  1067

cv.CREBα  tcggttttagGCTTATACAATAAAAGGAACACTTCCAATTGGTTTGGATAATACATCTTTAGCATCTCCTCATCAACTGGCTGAAGAAGCCACTAGAAAAAGGGAATTACGTCTTTATAA  1187
                    A  Y  T  I  K  G  T  L  P  I  G  L  D  N  T  S  L  A  S  P  H  Q  L  A  E  E  A  T  R  K  R  E  L  R  L  Y  K   200
hv.CREBβ            **C**C**************G*****G**G*****A********G***********G**C********C**A********T**G******C*C**G**************  628
                    A  Y  T  I  K  G  T  L  P  M  G  L  D  N  T  S  L  A  S  P  H  Q  L  A  E  E  A  T  R  K  R  E  L  R  L  Y  K   181
hv.CREBα            **C**C**************G***
                    A  Y  T  I  K  G  T  L

cv.CREBα  AAATCGgttagttttattttgtttcatatctgtggaaatattttataatatgaaatacttgattttaatttctttttaatagTGAAGCTGCTCGAGAAtgtcgaagaaaaaaaaaaagaata  1307
           N  R                                                                                                  E  A  A  R  E  C  R  R  K  K  K  E  Y   215
hv.CREBβ   ******                                                                                               ***G**C**CA*G******A**C*G*******C*C*TAC  673
           N  R                                                                                                  E  A  A  R  E  C  R  R  K  K  T  H  T   196

cv.CREBα  tgtaaagtgtctggagaatcgtgttgctgtactagagaatcaaaataaagctcttattgaagaacttaaatctttgaaagacttgtactgcagcaaaggagattaaattgtgctcaaaat  1427
           V  K  C  L  E  N  R  V  A  V  L  E  N  Q  N  K  A  L  I  E  E  L  K  S  L  K  D  L  Y  C  S  K  G  D  •••              249
hv.CREBβ  TGAAACTTTTTAGTCCCTGTGTAATAAGCATGATTTGCACAAAAAATTTAGTGGAAAGTGATTTACCTGAGTTTGTTTCATTACTGACAACATTGGATGCTCAGATCAAAGATTCAATAC  793
           E  T  F  •••

cv.CREBα  tacacagttctgttgtgtatttcatatctttacagttttaaagtgtagatatgatgcatgttcttgggaaatgtttaattcaaatcaaagtatttgtaaatataagaatagcattataaa  1547
cv.CREBα  ctctatgttaataaccagttcaacttttctgtttgaatatttttattccagaaataatttgtatacagttaaaagtttaacatttctttgcttttaaatgataaaataagaagcataaa  1667
cv.CREBα  aagtttcttatcccgaataatttatgcttcttatttttatctgagagatatttttttcactctgtttttatgtatttctttgcatttcttcgagggctttttcgtattaaaaattcaaaat  1787
cv.CREBα  attgaagtaatctatatagagctgacatctttggtttttcaaaataaatttttatttatgtatattgtatttatttaaagctattttatcagtggaaacaaaccttttataacacaaagca  1907
cv.CREBα  ggcatcaaatttattaatggactgatttattataatttcttttattcaaaaggataaacaagaaacaaacattgtaaggttttagcttaaagatctcttgtaaattgaaaattatagtt  2027
cv.CREBα  tatttttatatatgcttatttgaacgtgacttgtttttcaatttgtttgtgaggtttttgtagtttttcttttagttaaagctatatatttttcataaacatgtaaataactatgtaaaa  2147
cv.CREBα  aagcgttatcgaaacgtttccttgaatttttatgtcaggctgttccttttgcgtaggtatctatgaattattttaacgagattcgatttaaatttatgtatataacttttgtcttcatac  2267
cv.CREBα  caaggatcaactcttcgagttttttttttttttttcttccgtaatttaatctttattttcaagctagtaagtttaaataactgctaatgattgtataagtttatgttctatttaataattg  2387
cv.CREBα  actagctttttgtcaaatcagatgtgggttctttaggtctcaaatttatctattgttagtggacttcaacagcacattttatattcaagaatgttttcaaaacttcataattattatcga  2507
cv.CREBα  ataatttctaccgaaatttatgcgtagggtttgcataaaaaaaacttctctaagctaaaatttcttatttccttcaatagtattttcgtagtcttaaatttttatcataggaatgagatttt  2627
cv.CREBα  ccatagcttcttacggaagctt                                                                                                2645

hv.CREBβ  CTTATATAAATAGTCTACAGGAAATTTGGTGAAATATCTCGTGATGATGGGATGAGCTATCTTGAAACCAAATGTCATCTTTTTCTTGAGTATTTAATTGATGTTGCATATCTTATGTTA  913
hv.CREBβ  ATTAAAATGGATGGCAAGTCTCTGACAGATTATTCTTGTGTTGAGCGCCTTAGTTCGAACAAGAACTATTCTTGAAAAGATACGCCCAATTGACAAAAAGTTAACTTATCAAATTGATAA  1033
hv.CREBβ  ACTGATAAAAATGGCTTCCATTGAATCACTTAAAGGGATGTTCATCCTTTAAGCTTTAAGCCAAACGTTGATGATCTCATAACTAAAGAAGGTGCTAGTAGCGATGAAGATGAAAATGAT  1153
hv.CREBβ  GTATTAAAAGAAAAAGAAAGTATTCAGCAAGTTTATGTTCCGCCTAAAGTTACTGCTGTACCATATGATGATGATGACCGACTCTCTAAGAAGGAAAAAAGACTTCAGAAGTCCAAAAAA  1273
hv.CREBβ  CAGCTTTTGAGTAAAAGTTTACTTGAAGATTTGCGAACAGAGTACAGTGAGGCACCTGAAGAGATAAGCAGTAGGAGAAATAGCAAGAGAGCAAGAATCCAAGAAAAAGAAGAAGAAAGT  1393
hv.CREBβ  AGTTGTTTTGAGGAAGATAATTTGCGAAGACTTACG                                                                                  1429
```

B

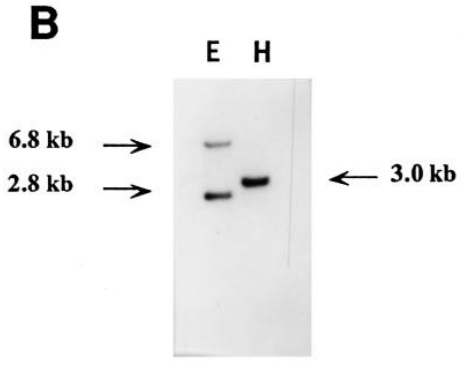

Fig. 5. (A) Hydra *CREB* gene sequence deduced from the cDNAs (upper case) *cv.CREBα*, *hv.CREBβ* and *hv.CREBα* and the genomic *cv.CREB* (lower case) sequencing. Stars indicate identical nucleotides between the cDNAs sequences. Numbering on the right is in italic for the nucleotides and in bold type for the amino acids. ••• indicate stop codons, exon/intron boundaries are underlined. (B) Genomic Southern blot of the cv.CREBα clone. Each lane contains 20 µg of cv. genomic DNA digested with *Eco*RI (E) or *Hin*dIII (H) and the blot was hybridized to a 615 bp long CREB cv. cDNA probe (from positions 421 to 1284).

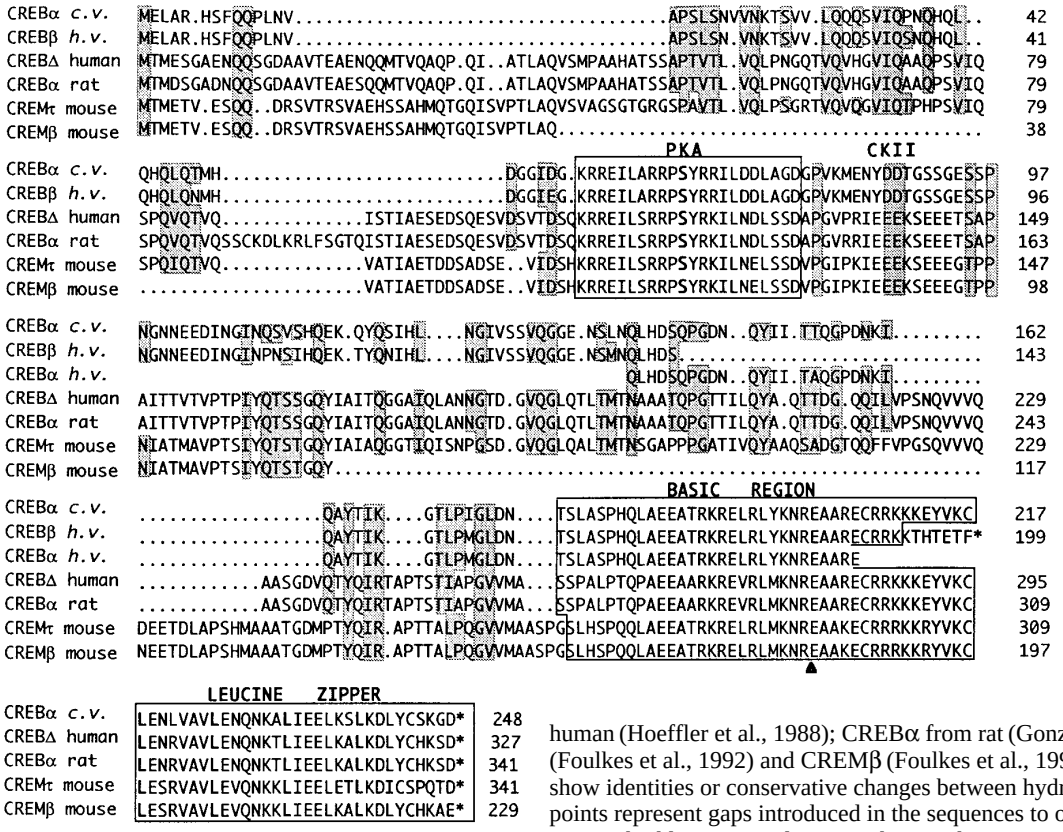

Fig. 6. Amino-acid sequence similarity between putative hydra CREB proteins and their vertebrate CREB and CREM counterparts. The predicted 248 amino-acid sequence of CREBα from *Chlorohydra viridissima (cv.)* is aligned with CREBβ (199 aa long) and CREBα (partial) from *Hydra vulgaris (hv.)*; CREBΔ from human (Hoeffler et al., 1988); CREBα from rat (Gonzalez et al., 1989); CREMτ (Foulkes et al., 1992) and CREMβ (Foulkes et al., 1991) from mouse. Shaded regions show identities or conservative changes between hydra and vertebrate sequences; points represent gaps introduced in the sequences to optimize the alignment. Boxes contain highly conserved regions that are the putative phosphorylation site for protein kinase A (PKA), the DNA-binding region (Basic Region) and the dimerization domain (Leucine Zipper). The inverted triangle under the basic region indicates the position of the conserved splice site.

The hydra CREB protein is involved in the CRE-binding complex

The full-length *cv.*CREBα protein expressed in a reticulocyte lysate system (Fig. 7A) is able to bind specifically to the CRE (Fig. 7B). A *cv.CREBα* cDNA was cloned into the pQE expression vector (Qiagen) and expressed in *E. coli.* Polyclonal anti-CREB antibodies were raised in rabbits against this hydra CREB protein and subsequently tested in western blots (Fig. 7C) and in band-shift assays (Fig. 7D). This antibody, when incubated with crude protein extracts, specifically recognizes two identical bands of about 32 and 35×10^3 M_r from *Hydra vulgaris* and *Hydra oligactis* but only the upper one from *Chlorohydra viridissima*. The same result was obtained when nuclear extracts were used in the western blot analysis (not shown). When this antibody was assayed in the band-shift assay (Fig. 7D) the following results were observed. In the presence of the preimmune serum, no modification of the migration of the CRE-binding complex is detected (Fig. 7D, lanes 7, 14, 21, 28 and 35) whereas in the presence of the immune serum a decrease in binding and a supershift of bands a and b are observed showing that the CREB protein is present and recognized in these CRE-binding complexes of the three hydra species tested, *Hydra vulgaris*, *Chlorohydra viridissima* and *Hydra oligactis*. Band c is similarly affected in *Hydra oligactis*. An excess of wt cold CRE DNA (Fig. 7D, lanes 6, 13, 20, 27 and 34) but not of the mutated version (Fig. 7D, lanes 5, 12, 19, 26 and 33) leads to the disappearance of this super-shifted complex proving thus that this complex which contains the CREB protein binds specifically to the CRE motif.

DISCUSSION

Head activator behaves as an agonist of the cAMP pathway in hydra

At the cellular level HA exerts three types of effects in hydra: it stimulates cells to divide and is responsible for the determination and the final differentiation of nerve cells and head-specific epithelial cells (Schaller et al., 1989). In hydra cells, two types of HA receptors are expressed, one with low affinity, which is most likely linked to the determination effect obtained with relatively high concentrations of HA (10^{-9} to 10^{-11} M) and one with high affinity, which triggers the mitogenic effect (Neubauer et al., 1990; Schaller et al., 1990). As we could detect an increase of cytoplasmic cAMP levels upon HA treatment only at concentrations higher than 10^{-11} M, we would expect this low affinity receptor to activate the cAMP pathway whereas the high affinity receptor may use some other signal transduction pathway.

Once the interstitial precursor cells are determined for the nerve-cell pathway, they become arrested in the G_2 phase. They will undergo mitosis and terminally differentiate only in the presence of the right signal: HA, FA or cutting-induced HI release (Hoffmeister and Schaller, 1987; Hoffmeister, 1991).

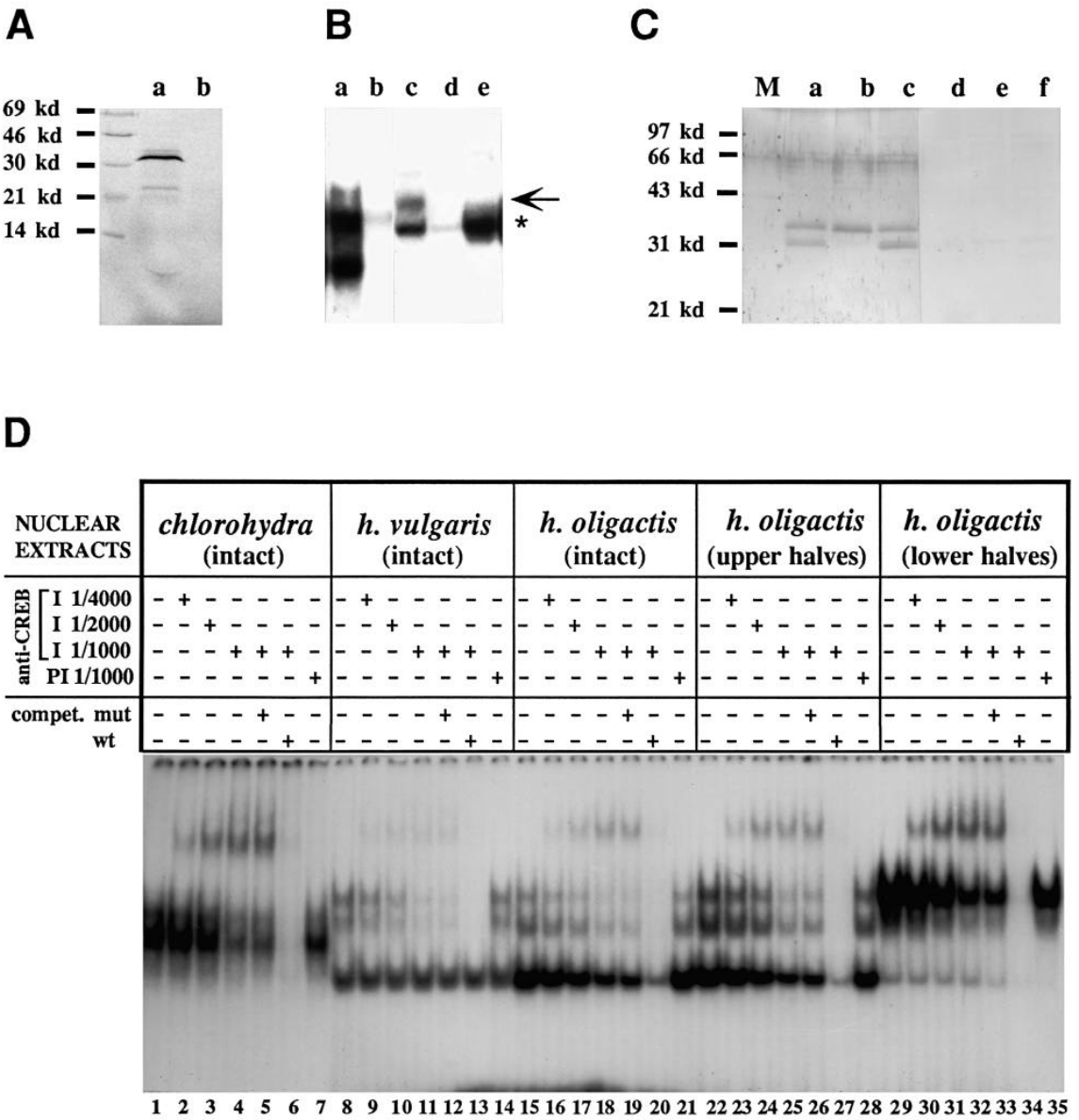

Fig. 7. (A,B) The in vitro translation product of the *cv.CREBα* binds specifically to CRE. A pBS plasmid encoding a *cv.*CREBα protein from positions 1 to 245 was constructed and one μg of this template was expressed in vitro in reticulocyte lysate by a coupled transcription-translation reaction in the presence of [^{35}S]met (Promega L4610). (A) 5 μl of the reaction was electrophoresed on a 12% SDS-PAGE. Lane a: plasmid carrying the *cv.CREBα* gene; lane b: plasmid without any insert. The *cv.CREBα* translation product migrates as a 32×10^3 M_r band (predicted 28). (B) Band-shift assay. 10 μg of *Chlorohydra viridissima* nuclear proteins (lanes a and b) or 3 μl of an unlabelled transcription-translation reaction of the *cv.CREBα* plasmid (lanes c and d) or the empty plasmid (lane e) were incubated in the presence of the CREwt element (lanes a,c,e) or the CREmut element (lanes b and d). Arrow: specific protein complex (lane c); asterisk: non-specific complex formed by lysate endogenous protein (lanes c,d,e). (C) Western analysis of the hydra CREB protein. 20 μg of crude protein extracts from *Hydra vulgaris* (lanes a,d), *Chlorohydra viridissima* (lanes b,e) or *Hydra oligactis* (lanes c,f) were incubated with the hydra CREB polyclonal antibody (lanes a,b,c) or the preimmune serum (lanes d,e,f). M: marker. (D) The rabbit polyclonal anti-hydra CREB antibody leads to a super shift of the hydra nuclear complex binding to the CRE. 10 μg of nuclear extracts of intact *Chlorohydra viridissima* (lanes 1-7), intact *Hydra vulgaris* (lanes 8-14), intact *Hydra oligactis* (lanes 15-21), upper (lanes 22-28) or lower halves (lanes 29-35) of *Hydra oligactis* 24 hours after cutting were used for this assay. I: immune serum collected after the third boost in lanes 2-6, 9-13, 16-20, 23-27 and 30-34. PI: preimmune serum in lanes 7, 14, 21, 28 and 35. Cold CREmut (lanes 5, 12, 19, 26, 33) or CREwt (lanes 6, 13, 20, 27, 34) DNAs were used as competitors in a 100× excess (1 pmole).

These signals lead 8 hours later to mature, recognizable nerve cells. As within this period of time the number of epithelial cells do not vary, the variations of the nerve over epithelial cells ratio reflect the intensity of the nerve-cell differentiation process. We could show that cAMP exposure can mimick this fast nerve-cell differentiation effect obtained with HA, FA or wounding.

Few molecular data are available about the extracellular signals and the transduction pathways they follow in order to achieve epimorphic regeneration processes. In the regeneration

of the newt limb, the proliferation of the blastema is nerve-dependent (Singer, 1974) and the mitogenic role of a purified neurotrophic factor has been demonstrated (Brockes and Kintner, 1986). Fluctuations of cAMP levels in blastemata during various stages of newt limb regeneration have been reported (Taban et al., 1978) and the observed increases were related to the cells directly and locally implicated in the limb regeneration process (Taban and Cathieni, 1989).

In hydra, several exogenous substances such as diacylglycerol or lithium have been shown to modify the positional value (Müller, 1989, 1991; Hassel and Berking, 1990) leading to multiheaded animals in the case of diacylglycerol (Müller, 1989) or to multiple ectopic feet after prolonged LiCl treatment (Hassel et al., 1993). In both cases, a modification in the inositol phosphate metabolism might be responsible for these alterations in the pattern formation (Berridge et al., 1989; Hassel et al., 1993), but no data is available, so far, about the regulatory endogenous signals targetting this signal transduction pathway.

The nuclear components of the cAMP transduction pathway are highly conserved in hydra

In mammals extracellular signals that target the cAMP second messenger are amplified by the stimulation of the cAMP-dependent protein kinase A (PKA), which in turn will activate the CREB transcription factor and its family members through phosphorylation. We have been able to isolate a hydra CREB-related gene whose sequence clearly indicates that the predicted hydra CREB protein is highly similar to the mammalian CREB/ATF family of transcriptional regulatory proteins but more closely related to the *CREB/CREM* genes. All the products of the mammalian CREB/ATF gene family interact with the same core promoter element (TGACGTCA) (Brindle and Montminy, 1992) through a bZip region. This hydra CREB protein shares extensive homology with the domains that have been proved to be required for CRE-binding and dimerization. From band-shift assays we could show that hydra nuclear proteins specifically bind to this motif and that, among this CRE-binding complex, the hydra CREB protein is recognized by a specific polyclonal hydra-CREB antibody. So far only three of the mammalian CREB/ATF gene family members have been shown to mediate a cAMP-dependent induction of transcription: CREB (Gonzalez and Montminy, 1989; Lee et al., 1990), CREMτ (Foulkes et al., 1992) and ATF1 (Rehfuss et al., 1991). From its highly conserved KID region one would predict that the hydra CREB protein may mediate such a cAMP-dependent induction of transcription.

From structural analysis, it is assumed that the two *CREB* and *CREM* genes have arisen by duplication of a common ancestor gene (Ruppert et al., 1992; de Groot and Sassone-Corsi, 1993). The hydra *CREB* gene might represent this common ancestor. As in its vertebrate cognates, the hydra *CREB* gene is multiexonic and encodes several isoforms. The exact conservation of the intron located between the basic region and the leucine zipper provides an additional argument for the common origin of the hydra *CREB* gene and the vertebrate *CREB/CREM* genes.

The hydra CRE-binding activity is modified during regeneration or after HA exposure

Three different specific retarded bands were identified when nuclear extracts from *Hydra oligactis* or *Hydra vulgaris* were incubated in presence of the CRE motif. During regeneration, we observed dramatic and specific differences in the abundance of these complexes binding to the cAMP response element compared to their steady-state level in intact animal. As we have shown that the CREB protein, which requires dimerization for DNA-binding, is found in these three complexes, we postulate that each of them might reflect a different in vivo composition leading to a different transcriptional transactivation potential. Two mechanisms might then explain the changes in the CRE-binding pattern observed during regeneration. First, regulation of dimerization through formation of various homo- or heterodimers would favor the formation of the largest complex (band a) and thus modify the abundance of each of these complexes; second, due to post-translational modifications, the DNA-binding affinity of the largest complex would be enhanced during regeneration. Both mechanisms might also be combined.

Regulation of DNA-binding activity of transcription factors by extracellular signals through phosphorylation has been documented for several transcription factors (reviewed by Hunter and Karin, 1992). In the case of the *CREB/CREM* genes, PKA activity can affect the CRE-binding activity of CREB (Nichols et al., 1992) but not that of CREMτ (de Groot et al., 1993) whereas the CRE-binding of both CREB and CREMτ have been shown to be modulated by others kinases (Kramer et al., 1991; de Groot et al., 1993). The data presented in this paper suggest that the HA-induced cAMP increase correlates with a modification in the CRE-binding activity of the hydra CREB protein.

The fact that variations in the CRE-binding activity during HA exposure (Fig. 4) are greater with the ATF site than with the CRE site is in agreement with the observation that the former, asymetric, behave as weak binding sites and the latter, symetric, as strong ones (Nichols et al., 1992). Thus the asymmetric CREs show a lower basal level but a higher fold of stimulation by cAMP than the symmetric CREs (Nichols et al., 1992). We are currently investigating such asymetrical CREs present in the flanking sequences of the hydra *cnox2* gene which have been shown to exhibit different levels of transcripts during regeneration (Schummer et al., 1992).

Modifications in the CRE-binding activity are concomittant to nerve-cell differentiation and cell determination in hydra

From band-shift experiments, we have shown that HA treatment leads to alterations of the CRE-binding pattern of nuclear proteins from intact hydra. As a growth factor HA elevates the mitotic index of HA-treated hydra within 1-2 hours at concentrations as low as 10^{-13} M (Schaller et al., 1989). As in our experiments, HA at 10^{-13} M was unable to modify either the CRE band-shift pattern nor the cAMP levels, it seems very unlikely that this mitogenic effect of HA is mediated by cAMP.

We have shown that the CRE-binding activity is strongly increased as soon as 2 hours after initiation of regeneration by cutting. Wounding hydra induces a local release of HA (Holstein et al., 1986; Hoffmeister and Schaller, 1987) and we assume that the alterations in the CRE-binding activity detected during regeneration rely at least partly on this HA release. During this time a general drop of cells in mitosis is observed (Park et al., 1970; Holstein et al., 1991), recovering

slowly during the next 24 hours. As a specific and significant change in cell distribution an increase in nerve-cell density occurs at the regenerating tip, independent of whether the animals regenerate head or foot. This increase is due to differentiation of cells determined for the nerve-cell pathway, arrested in G_2. Treatment with HA or FA or wounding relieves this arrest and, after a final mitosis, leads, 6-8 hours later, to the appearance of new mature cells. This final mitosis occurs at a time when other cells remain blocked, suggesting differential regulation. We propose that stimulation of this final mitosis and terminal differentiation to nerve cells is mediated by cAMP as second messenger and CREB as transcription factor.

This work provides the first evidence showing that in hydra cells, cAMP is one of the second messengers targeted by HA and, as predicted from similarities between yeast and vertebrate, nuclear components from the cAMP pathway, namely the cAMP response element and the CREB transcription factor, are structurally and functionally conserved in such a phylogenetically primitive animal as hydra. Further investigations should demonstrate how the regulation of the CRE-binding activity modulates the transcriptional activity of genes involved in regeneration and/or HA response.

We thank Stefanie Waldmann for her excellent technical assistance, Sylvia Kreger and Birgitta Schinke for their daily care of the hydra culture and Carole Seum-Oyneser for her help with the western analysis. We are very grateful to Michael Boshart and Laura Runkell for their helpful comments throughout this work and their critical reading of the manuscript, and to Bob Hipskind for technical advice. This work was supported by grants from the Bundesministerium für Forschung und Technologie (BMFT, BCT 365/1), the Swiss National Science Foundation and the Claraz Foundation.

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(Accepted 18 January 1995)

Note added in proof

The accession numbers are: X83872 for the *Hydra vulgaris* mRNA for cAMP response element binding protein gene, and X83873 for *Chlorohydra viridissima* CREB gene.