Volume 13 Number 17 1985 Nucleic Acids Research

Cloning of the chick hsp 90 cDNA in expression vector

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Received 12 July 1985; Accepted 6 August 1985

ABSTRACT

A cDNA clone for the 90kDa heat-shock protein, which we have recently identified as a component of steroid hormone receptors in their heteromeric 8S form, was isolated by direct immunological screening of a chicken smooth muscle cDNA expression library, prepared in the expression plasmids pUC8 and pUC9. Using polyclonal and monoclonal antibodies against the 90kDa protein a colony was identified that reacted with both antibodies. Plasmid 9.11 (p9.11, ~ 1100 base pair insert) was found to hybrid-select mRNA for the 90kDa heat-shock protein. Northern blot analysis revealed that RNA isolated from various chicken tissues contain a single transcript of ~ 3 Kb hybridizing to a [^{32}P]labelled cDNA probe made from p9.11. Heat-shock treatment of chick embryonic fibroblasts resulted in increased steady-state levels of a 3 Kb transcript in both poly A+ and poly A- RNA fractions. Southern blot analysis of chicken genomic DNA indicated that the cDNA hybridizes to a single copy sequence. Sequence data show that the p9.11 cDNA displays a high degree of homology with the 5' portion of yeast heat shock protein 90 cDNA.

INTRODUCTION

Steroid hormone receptors found in the cytosol of target cells have an approximate 8S-sedimentation coefficient corresponding to an oligomeric structure with a molecular mass of 250-300kDa (1-4). This oligomeric complex contains a non hormone binding 90kDa component recognized by the BF_4 monoclonal antibody (5-7). The 90kDa protein is more abundant (~ 100 times) than the steroid hormone binding subunits and is present in all tissues of the chick (8, 9). Recently we have found that the 90kDa protein is identical with the 90kDa heat-shock protein (hsp 90) or stress protein (10). No function is known for hsp 90, a protein widely conserved among species (11, 12), expression of which is regulated during development (13) and by a variety of stress conditions (14). Only the yeast hsp 90 cDNA (15) and the Drosophila hsp 83 gene (16) have been cloned. Comparison of hsp 90 cDNA sequence of different species as well as of gene organisation with its regulatory elements, may help in further analysis of the role of hsp 90 and

of its association with steroid hormone receptors and with oncogene products (17, 18).

Here we describe the identification of cDNA clones coding for the chicken hsp 90 by direct immunological screening of a chicken cDNA expression library. In addition, expression of the 90kDa related sequences in a variety of chick tissues, as well as in heat-shock treated cells, was studied. High homology between the yeast and chicken hsp 90 cDNA has been found.

MATERIALS AND METHODS

Isolation of cDNA clones

Preparation of the chicken smooth muscle cDNA expression library is described in detail elsewhere (19). Smooth muscle poly(A)+ RNA used for cDNA synthesis was prepared from embryonic (11 day old) chicken stomachs and gizzards. Replica filters (20) containing bacterial colonies were screened using the immunological procedure described (19). Antibodies were partially purified by two ammonium sulphate precipitations (40 %) of ascitic fluid or serum followed by dialysis against PBS (protein concentration : ~ 5 mg/ml). The monoclonal antibody (BF_4) utilized was a rat IgG2b (5) recognizing only the 90kDa protein in soluble extracts of all chicken tissues by Western blots analysis (8). Antibodies to the 90kDa protein (B90) were also purified from the goat serum IgG-G_3 (7, 21) by affinity chromatography on 90kDa purified chicken protein (10) covalently coupled to Sepharose 4B (Pharmacia). The BF_4 and the B90 antibodies were utilized for bacterial colonies screening, at a dilution of 1:2000 and 1:50 respectively, for an incubation time of 2 h. The second antibodies, iodinated by cloramine T method (22) were rabbit anti-rat or anti-goat IgG and were incubated with the filters for 1 h. Potential positive colonies were reisolated, rescreened with both antibodies (2nd screen) and than tested by translation of hybrid selected mRNA.

Hybrid-selection of mRNA

Plasmid DNA from clones identified by antibody screening was prepared following a described procedure (23). Binding of DNA to nitrocellulose, hybridization and elution of mRNA were performed according to Parnes (24). Twenty µg of plasmid DNA were fixed onto 16 mm^2 of nitrocellulose and hybridized with 10 µg of poly A+ chick oviduct mRNA. Both mRNA remaining in hybridization solution and the mRNA eluted from the filter were translated *in vitro*.

Cell free translation and immunoprecipitation of 90kDa protein

Cell free translation (25) in a nuclease treated rabbit reticulocyte lysate (Amersham) containing [^{35}S] methionine (1.200 Ci/mmol, 1 Ci = 37 G bq ; Amersham) was carried out at 30°C for 90 min. The lysate was subsequently incubated overnight at 4°C with 3 µl of IgG-G_3 antibody (5 mg/ml). Immunocomplexes were precipitated by addition of 50 µl of rabbit antigoat IgG (5 mg/ml) (1 h of incubation at 24°C) and then centrifuged twice (10.000 g) through a cushion of 1 M sucrose, 1 % DOC, 150 mM NaCl in 10 mM Na phosphate buffer pH 7.4. The pellet was dissolved in Laemmli sample buffer and analyzed on a Na Dod SO_4/polyacrylamide gel (26). Protein bands were detected by fluorography at - 70°C.

RNA preparation and Northern blot analysis

Total RNA from various chick tissues or from chick embryo fibroblasts was prepared by LiCl-urea method (27). The poly A+ mRNA was obtained after two cycles of oligo(dT)-cellulose chromatography (28), RNA was electrophoresed through agarose gel containing 2.2 M formaldehyde, then transferred to nitrocellulose and hybridized to [^{32}P] labeled cDNA insert (29, 30). The specific activity of the nick translated probe was routinely 4-6 x 10^8 cpm/µg.

DNA sequencing

DNA was sequenced by the dideoxy method essentially as described (31), except that [α^{35}S]dATP was used as the label. Appropriate restriction fragments were subcloned into M13 mp8/9 phage (32).

RESULTS

Identification of clones encoding chicken hsp 90

In order to identify recombinant clones encoding the 90kDa heat-shock protein, a 20,000 member cDNA expression library (19) was screened for bacterial colonies producing proteins antigenically related to chicken hsp 90.

Half of the library was screened with the BF_4 monoclonal antibody, the other half with the B90 polyclonal antibody. Twenty potentially positive colonies (15 from pUC 9 and 5 from the pUC 8) were rescreened with both antibodies. One colony from the pUC 9 (9.11) clearly expressed antigens detected by both antibodies and one colony from the pUC 8 (8.1) was found to react weakly only with the monoclonal antibody. The experiment with the monoclonal antibody is shown in Fig. 1. Plasmids from 9.11 and 8.1 colonies (p9.11 and p8.1) were isolated and found to contain cDNA inserts of 1.1 and

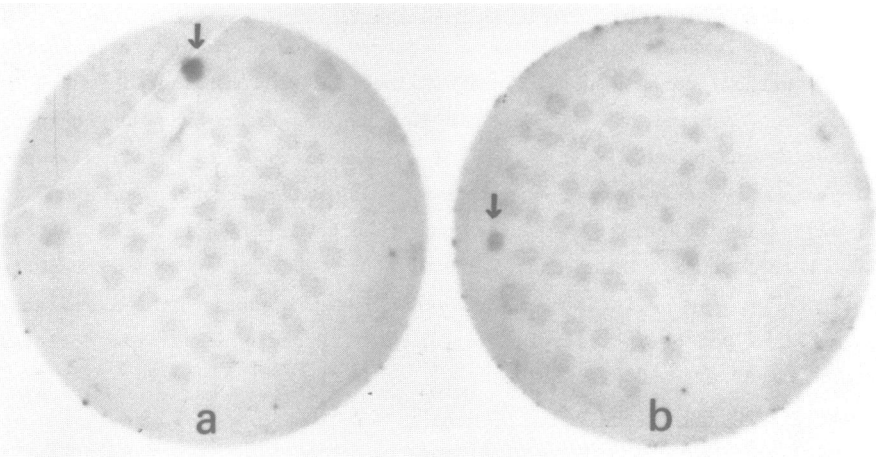

Fig. 1 : Second bacterial colonies screen with BF_4 antibody.
Filters a and b contain colonies from the pUC9 and pUC8 library respectively.
The BF_4 positive colonies 9-11 (a) and 8-1 (b) (arrows) were amplified for further analysis.

1.3 Kb, respectively. To determine whether these cDNA inserts encoded sequences for hsp 90, hybrid-selection translation was carried out using poly A+ RNA from oestrogen-stimulated chick oviduct. This is the tissue from which we initially purified and identified the chicken hsp 90 with the BF_4 antibody (10). The immunoprecipitation with $IgG\text{-}G_3$ antibody of *in vitro* translation products of mRNA selected by p8.1 and p9.11 DNA are shown in Fig. 2 (lane 3 and 4) : only p9.11 DNA-selected messenger yielded a protein product with an apparent molecular weight of 90,000. In one-dimensional gel electrophoresis it migrated similarly to the BF_4-immunopurified hsp 90 (Comassie blue staining, lane 5'). For comparison, immunoprecipitation of translation products of poly A+ oviduct mRNA (1 µg) are shown in lane 2. The crude antibody $IgG\text{-}G_3$ immunoprecipitates other proteins among the translation products of total mRNA and the labelled band at ~ 43,000 Da probably represents the non-glycosylated translation product of ovalbumin mRNA, the most abundant mRNA (~ 50 %) of estrogen stimulated oviduct (33). No 90kDa immunoprecipitable translation product was obtained after hybrid-selection with the p8.1 DNA filter (lane 3). Lanes 6 and 7 show that the mRNA coding for a 90kDa protein is still present in the hybridization medium of p8.1 and that an important decrease of the same translatable mRNA is observed in hybridization medium of p9.11. Subsequently the p9.11 insert was not found

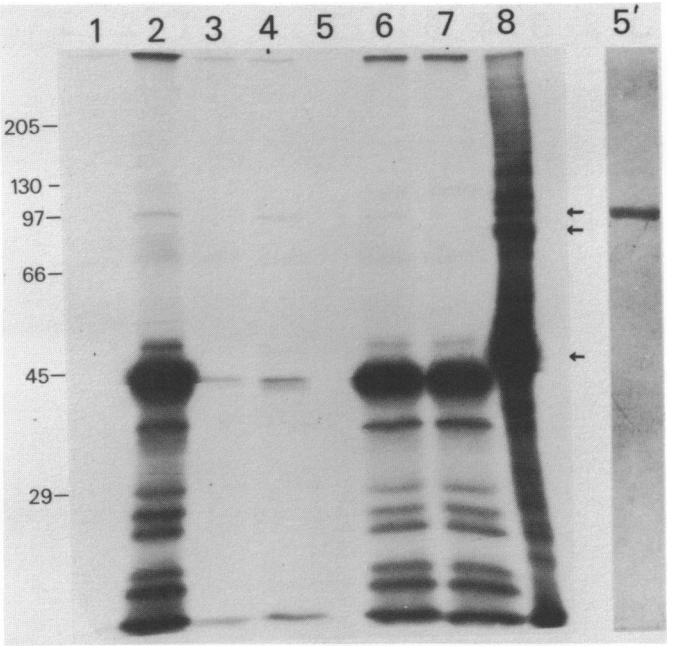

Fig. 2 : Immunoprecipitation of translation products of mRNA hybrid-selected by p9.11 and p8.1 plasmids. The hybrid selection, in vitro translation in the presence of [^{35}S] methionine and immunoprecipitation were carried out as described in Materials and Methods section. All translation samples were immunoprecipitated by IgG_3 antibody before SDS-PAGE analysis (10 %), Comassie blue staining and fluorography :

Lane 1 : control of translation with H_2O instead of added messenger.
Lane 2 : translation products of 1 µg of oviduct mRNA.
Lane 3 : translation of mRNA hybrid-selected by p8.1 DNA.
Lane 4 : translation of mRNA hybrid-selected by p9.11 DNA.
Lane 6 : translation of mRNA not hybridized to p9.11 DNA.
Lane 7 : translation of mRNA not hybridized to p8.1 DNA.
Lane 8 : pattern of chick oviduct proteins labelled in tissue explants in vitro with [^{35}S] methionine during 30 min. Arrows indicate the migration of 90kDa protein, conalbumin (76kDa) and ovalbumin (43kDa).
The lane 5' shows the Comassie blue stained immunopurified hsp 90.
Molecular weights of marker proteins (kDa) are indicated on the left.

to hybridize with the p8.1 insert (data not shown).

Detection of mRNA coding for the 90kDa hsp in different chick tissues

To identify the transcript encoded by p9.11 insert, RNA from various embryonic or adult chicken tissues (Fig. 3a, 3b) or from secondary culture of chick embryo fibroblasts (Fig. 4a) maintained at a normal or elevated temperature (i.e. heat-shocked for 3 h at 44°C) were analyzed by Northern blotting. The nick-translated insert of p9.11 clone revealed in RNA from all tissues an ~ 3 Kb messenger. Moreover a dramatic increase of the 3 Kb mRNA

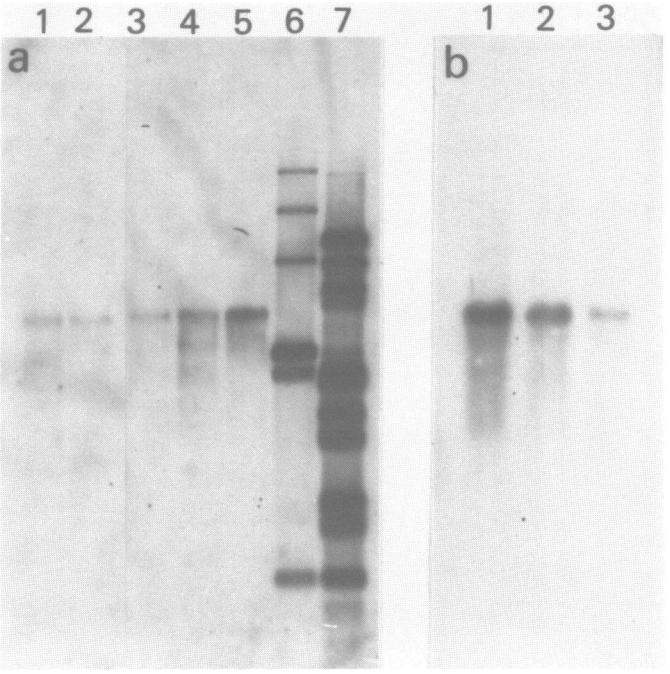

Fig. 3 : Presence of hsp 90 in different tissues of the chicken.
Poly A+ RNA samples were analyzed by the Northern blot method with the p9.11 [^{32}P] labelled as probe.
a) 1 µg of mRNA from embryonic chick tissues : lane 1 : smooth muscle, lane 2 : striated muscle, lane 3 : liver, lane 4 : brain, lane 5 : fibroblasts. Markers : lanes 6 and 7 λ phage cut with Hind III and Hind III plus EcoRI respectively.
b) adult chick tissues. Lanes 1 and 2 : oviduct mRNA, 12 and 4 µg respectively ; lane 3 : 4 µg of liver RNA.

was observed after heat-shock of chick embryo fibroblasts in both poly A+ and poly A- RNA fractions with an additional hybridization signal at 2.5 Kb (Fig. 4a).

Increased levels of hsp 90 mRNA have been already reported for heat-shocked Drosophila cells (34, 35) and canavanine treated chicken embryo fibroblasts (36). The heat-shock regulation of hsp 90 mRNA levels in chick embryo fibroblasts seems to affect both poly A+ and poly A- fractions while in the Drosophila cells a greater difference at the level of polyadenylated messenger was observed (34, 35). The distribution of hsp 90 mRNA in poly A+ and poly A- fractions was monitored also in chick oviduct RNA and compared to the distribution of c-myc mRNA (37). Figure 4b shows that the 3 Kb mRNA

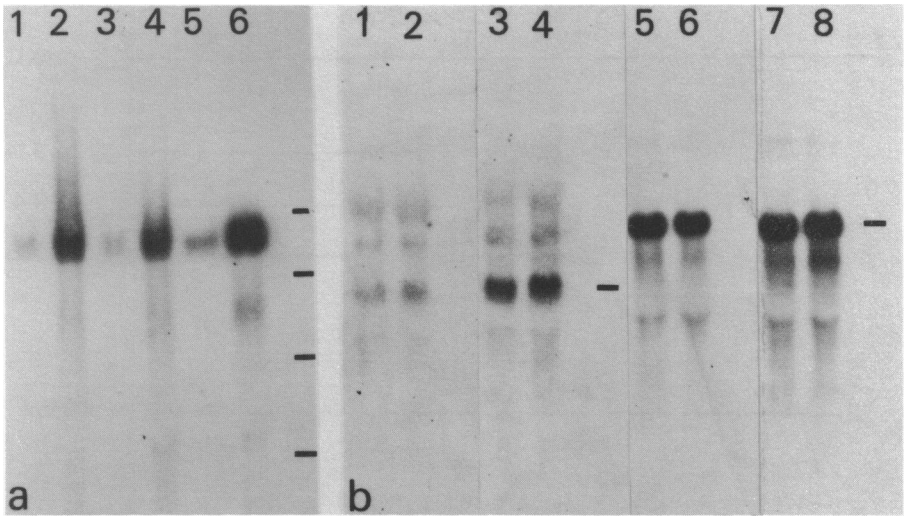

Fig. 4 : hsp 90 mRNA induction by heat shock.
a) Chicken fibroblasts were kept at 37°C (lanes 1, 3, 5) or incubated 3 h at 44°C (lanes 2, 4, 6).
Total RNA (lanes 1 and 2 : 20 µg each), poly A- RNA non retained by oligo (dT) (lanes 3 and 4 ; 60 µg each) and poly A+ RNA (lanes 5 and 6 ; 5 µg each) were electrophoresed blotted, and hybridized with the p9.11 probe. Lines on the right show size markers of 3.2, 2.7, 2.2 and 1.7 kb.
b) Two independent samples of total RNA (15 µg, lanes 1, 2, 5, 6) and of poly A+ RNA (10 µg, lanes 3, 4, 7, 8) from chicken oviduct were analyzed. The blots were hybridized with the p9.11 probe (lanes 5 to 8) and then rehybridized with the c-myc probe (lanes 1 to 4).

signal is approximately equally represented in total and in poly A+ fractions, whereas the 2.4 Kb c-myc mRNA signal was enriched by oligo-dT cellulose chromatography when compared to total RNA.

Expression of antigenic determinants recognized by BF4 and B90 antibodies

By rescreening (38) the entire 100,000 member smooth muscle pUC8/9 cDNA library (39) with the 1.1 Kb purified insert of p9.11 plasmid, 7 other positive clones were identified. Three independent positive clones in the pUC9 library (9.10, 9.13 and 9.17) had an insert of the same size and with the same restriction map as the p9.11. Clones with smaller inserts, either in pUC8 or in pUC9, were identical on the basis of the restriction maps starting from the 3' portion of the p9.11 cDNA. It appears, therefore, that all inserts are comprised in the p9.11 cDNA as shown in Figure 5. Only the 4 clones with the 1.1 Kb insert from the pUC9 library expressed a fusion protein recognized by the two antibodies (Fig. 5a,b). We concluded that for

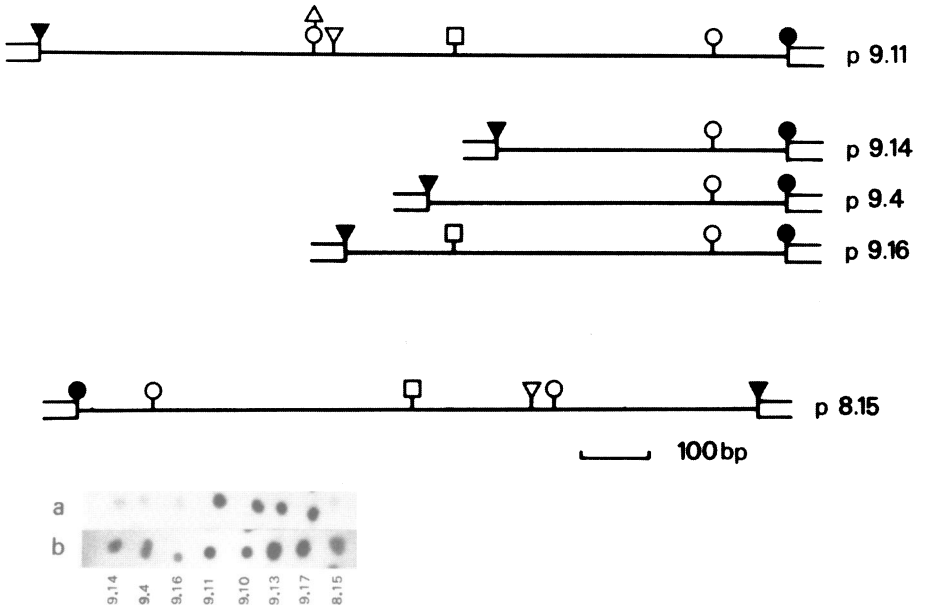

Fig. 5 : Restriction maps of the hsp 90 cDNA clones.
Restriction maps of cDNAs isolated from pUC8 and pUC9 libraries have been obtained with the following enzymes : Sal I (▼), Eco RI (●), Rsa I (o), Kpn I (Δ), Pst I (▽), Pvu II (□). In p8.15, the Kpn I enzyme was not tested.
Bacterial colonies containing the indicated plasmids were tested for expression of the hsp 90 antigenic determinants with the monoclonal (BF_4) antibody (a), and with the polyclonal (B 90) antibody (b).
Clones 9.10, 9.13 and 9.17 are identical to the 9.11.

expression of the antigenic determinant the insertion of p9.11 cDNA in pUC9 is correct in the orientation. Comparison of restriction maps of hsp 90 clones identified by colony hybridization (Fig. 5) with the purified 1.1 Kb insert of p9.11 and the frequency by which we found the same 1.1 Kb insert in independent positive colonies (4/8) suggested that both restriction sites utilized in cloning procedure are internal restriction sites of the hsp 90 cDNA.

hsp 90 gene complexity

The cDNA insert of p9.11 was labelled by nick-translation and used to detect hsp 90 genes by hybridization to Southern blots (40) of restriction endonuclease digests of genomic DNA. Results presented in Figure 6 indicated that the hsp 90 chicken gene is a unique split gene.

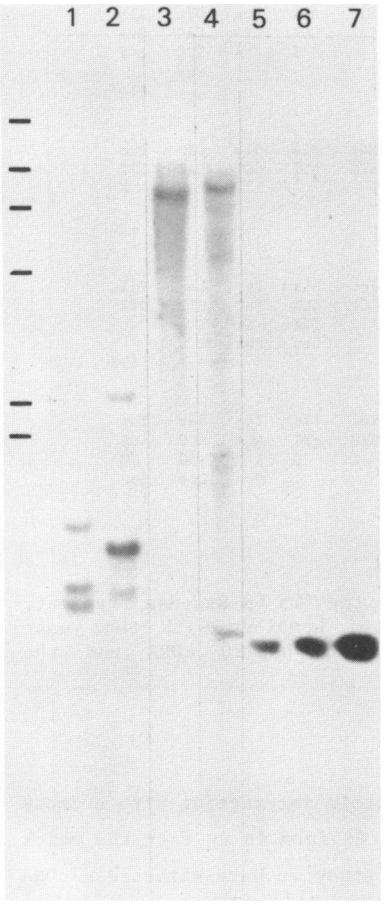

Fig. 6 : Complexity of hsp 90 gene.
Fifteen µg of genomic DNA were digested with Pst I (lane 1), Pvu II (lane 2), Bam HI (lane 3), Kpn I (lane 4).
For approximative estimation of gene copy number, different quantities of the p9.11 insert were run in lanes 5 (7.5 pg), 6 (15 pg) and 7 (30 pg) corresponding to 0.5, 1 and 2 gene equivalents.

Homology between chicken and yeast hsp 90

In order to confirm that the p9.11 insert codes for the hsp 90, a portion of cDNA was sequenced after subcloning the Rsa-Rsa fragment of ~ 525 nucleotides in M13 mp8 vector. The cDNA sequence of 120 nucleotides and the derived aminoacid sequence is shown in Figure 7 and compared to the homologous cDNA sequence of yeast hsp 90 cDNA (15). High degree of homology exists also starting from aminoacid 103 of Drosophila hsp 83 (41). Considering the high degree of conservation of hsp 90 among the species, this result allows us to conclude that the chicken p9.11 cDNA corresponds to the 5' portion of hsp 90 coding sequence.

	Thr	Lys	Ala	Phe	Met	Glu	Ala	Leu	Glu	Ala
Chicken	ACC	AAG	GCT	TTC	ATG	GAA	GCA	CTG	CAG	GCA
Yeast		A	C				T	A	TCT	T
									Ser	
	101									110
	Gly	Ala	Asp	Ile	Ser	Met	Ile	Gly	Gln	Phe
Chicken	GGG	GCT	GAT	ATT	TCC	ATG	ATT	GGT	CAG	TTT
Yeast	T	C		G A					A	C
				Val						
	111									120
	Gly	Val	Gly	Gly	Tyr	Ser	Ala	Tyr	Leu	Val
Chicken	GGT	GTT	GGT	TTC	TAC	TCT	GCT	TAC	CTT	GTT
Yeast				T			TTA	T	T A	
				Phe			Leu	Phe		
	121									130
	Ala	Glu	Lys	Val	Thr	Val	Ile	Thr	Ser	Thr
Chicken	GCG	GAG	AAG	GTG	ACA	GTG	ATC	ACA	AGC	ACA
Yeast	C	C	GA	T	CAG	T	T	T	AG	GC
		Asp	Arg		Gln			Ser	Lys	Ser
	131									140

Fig. 7 : Partial sequence of the p9.11 insert.
The sequence corresponds to the 5' end of the 525 bp Rsa-Rsa fragment. The orientation was inferred on the basis of homology with the yeast hsp 90 cDNA. The differences between the chick hsp 90 cDNA and the yeast hsp 90 cDNA are shown.

DISCUSSION

We have recently found that the 90kDa protein interacting with hormone binding subunits of steroid receptors in their 8S form is in fact the chick 90kDa heat-shock protein (10). In the present study we have screened a cDNA expression library with antibodies originally raised against the 8S form of chicken progesterone receptor. The monoclonal antibody (BF_4) was specific for the 90kDa protein and the polyclonal antibody ($IgG\text{-}G_3$) was made monospecific for the same protein by affinity chromatography purification of the serum. Direct immunological screen of a smooth muscle cDNA expression library allowed the isolation of p9.11 cDNA (~ 1.1 Kb) which hybrid-selected the hsp 90 mRNA.

Northern blot analysis of RNA from various embryonic or adult chick tissues revealed a transcript of ~ 3 Kb. This 3 Kb messenger was dramatically increased in both poly A+ and poly A- RNA fractions by heat-shock of chick embryo fibroblast secondary cultures. Regulation either at the transcriptional level or at the level of mRNA stabilization, may account for the increased amount of hsp 90 mRNA after heat-shock. Our observation that

hsp 90 mRNA existed in both poly A+ and poly A- forms agrees with those of Storti et al. (34) and O'Connor (35). However in Drosophila cells a dramatic increase of only polyadenylated fraction of translatable hsp 83 mRNA after heat-shock has been described. Similar results based on DNA-RNA hybridization were also reported by O'Connor and Lis (35). Evidently, the mRNA molecules not retained on the oligo(dT) cellulose column may still possess a very short poly A sequence.

In the construction of the cDNA expression library that we have screened, two different synthetic linkers (Eco RI and Sal I) were sequentially added to cDNA molecules before insertion into the pUC8/pUC9 vectors (19, 39). With this method one bacterial clone in three should possess a cDNA capable of expressing a portion of the protein for which the template mRNA normally codes. In this study we utilized an immunological screening procedure followed by use of a [^{32}P] labelled probe. Having found 4 clones in the pUC9 with the same insert (1.1 Kb), all expressing antigens related to chicken hsp 90, and one clone (p8.15) in pUC8 with ~ 1 Kb insert also contained in the p9.11 with an opposite orientation, we believe that both cloning sites (Sal I and EcoRI) are also internal sites in the hsp 90 cDNA. Antigenic determinants related to hsp 90 are found only on pUC9 clones with a cDNA insert of 1.1 Kb and not on pUC9 smaller clones. Although the hsp 90 is a protein widely conserved among species (11, 12), the BF_4 antibody does not cross react with mammalian or amphibian hsp 90 (M.G. Catelli, unpublished data). A portion of cloned cDNA may be a region more divergent among the species than others and indeed responsible for the expression of antigenic determinant recognized by BF_4 antibody. Partial sequence data and comparison with yeast hsp 90 DNA sequence (15) and Drosophila hsp 83 DNA sequence (41) indicated that we cloned the 5' portion of the coding sequence and reinforced the conclusion that both restriction sites utilized in the cloning procedure are internal cDNA restriction sites. Only the sequence of the full-length cDNA (which we are at present isolating with heterologous (35) probe from the same library) may clarify the degree of homology with hsp 90 gene from other species. In addition, experiments where synthesis of the hsp 90 may be regulated in cultured cells (transfection of constructs expressing the protein) will be made possible when full length cDNA will have been cloned. Such experiments may help for understanding the role of the hsp 90 including its interaction with oncogene products (17, 18, 42) and with steroid hormone receptors (6, 10).

ACKNOWLEDGEMENTS

We thank Etienne Emile Baulieu for support and encouragement of this work.

We thank Michel Renoir, Irene Joab and Christine Radanyi for providing IgG-G_3 antiserum and BF_4 hybridoma cells, and Ingrid Jung-Testas for preparing secondary cultures of chick embryo fibroblasts.

We are also grateful to Beatrice Chambraud and Jan Mester for helpful discussion and to Martine Rossillon, Françoise Boussac, Luc Outin, Jean-Claude Lambert for preparation of the manuscript and figures.

Bacterial colonies were stored and replicated in the facilities of Inserm's SC30 laboratory.

This work was supported in part by grant ATP 03449 (MGC) from the Centre National de la Recherche Scientifique (France) and by NIH grant CA 29569.

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