molecules could be observed (Fig. 3*c*) and measurement indicated that these structures were formed by contacts between receptors rather than by a single receptor oligomer (not shown). Identical structures were seen with DNA fragments of the uteroglobin gene carrying only one HRE (Fig. 3*d*–*f*). The precise measurement of the apparent length of DNA before and after binding receptor was possible with these short DNA fragments; no significant differences were found, suggesting that no complex DNA structures were generated by binding to receptor.

At present there is no direct experimental evidence relating receptor–receptor interaction to hormone function. It should be noted, however, that in nearly all steroid-regulated genes there are several receptor binding sites[5,7,8,10,11] which could facilitate the association of receptor oligomers. Moreover, the presence of both receptor binding sites upstream from the MMTV[12,13] and chicken lysozyme[14] genes is necessary for optimal hormonal activity. Loop formation may allow binding of transcription factor(s) on the loop DNA (Fig. 4*a*) or the loop may not itself be important but simply be a consequence of receptor–receptor interaction (see Fig. 1, *c*–*f*), which in turn modulates the activity of a transcription factor (Fig. 4*b*). This could explain the location of HREs at a distance from the promoter region of the gene. Cordingley *et al.*[15] have recently shown that glucocorticoid receptor binding to the HREs in MMTV is accompanied by the binding of two proteins in the promoter region, one of which is the nuclear factor 1. It is not known whether steroid receptor binding to DNA is a cooperative process when several HREs are present on the same DNA molecule.

Several steroid receptor genes have now been cloned[16–18] which allows *in vitro* mutagenesis to locate the inter-receptor binding regions and correlate these with biological activity.

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Heat shock factor is regulated differently in yeast and HeLa cells

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When cells are exposed to elevated temperatures, transcription of a small set of genes, the heat-shock genes, is activated[1–5]. This response is mediated by a short DNA sequence, the heat-shock element (HSE)[5–7], which is thought to be the binding site for a specific transcription factor[8–12]. Studies with Drosophila show that this protein binds to HSEs only in heat-shocked cells, suggesting that changes in factor binding are responsible for gene activation[10]. We have investigated the properties of HSE-binding proteins from yeast and HeLa cells. In HeLa cells, binding activity is present only after heat shock. In contrast, control and heat-shocked yeast cells yield the same amount of HSE-binding activity; however, the mobility of protein-HSE complexes on polyacrylamide gels is altered following heat shock. This mobility difference can be significantly reduced by treatment of crude extracts with phosphatase. We propose that the yeast heat-shock factor binds constitutively to DNA but only activates transcription after heat-induced phosphorylation.

We looked for proteins in HeLa cells and yeast (*Saccharomyces cerevisiae*) with the ability to bind to a synthetic HSE sequence[5,7]. This HSE matches a consensus derived from a variety of eukaryotic heat-shock promoters, and is functional in mammalian[7], *Xenopus*[7], *Drosophila*[13] and yeast[12] cells. Figure 1 shows the results of binding assays using a probe that contains two overlapping consensus HSEs (this appears to be an optimal binding site *in vivo*)[5]. Binding proteins were detected by their ability to form complexes with labelled DNA and thus retard its migration on polyacrylamide gels[14,15]. Heat-shocked HeLa cells contained such a protein, and binding could be abolished by competition with excess unlabelled probe (Fig. 1*a*). With a small amount of extract two labelled bands were visible, but at higher protein concentrations only one slowly migrating band was seen (Fig. 1*b*).

Yeast extracts contained a similar activity, and again binding could be abolished by addition of excess unlabelled probe to the binding reaction (Fig. 1*a*). To compare the sequence specificities of the HeLa and yeast binding proteins, we performed binding assays in the presence of various unlabelled competitor DNAs: a monomeric HSE, a tetrameric HSE, and a dimeric HSE with a mutated sequence that matches the consensus in only six out of eight positions[7,12]. Both yeast and HeLa proteins bound the tetramer more strongly than the monomer, and the mutant HSE (HSE12) was the weakest competitor of all (Fig. 1*c*). Competition by HSE12 was detected with the HeLa extract but not with the yeast extract. This correlates with the ability of HSE12 to support transcription in heat-shocked cells: it is active in mammalian cells[7], but completely inactive in yeast[12]. As a further comparison of the yeast and HeLa proteins, we performed DNase I protection experiments using a longer probe containing the synthetic dimeric HSE. As shown in Fig. 2, identical footprints were obtained with each extract. These experiments establish that yeast and HeLa cells contain HSE-binding proteins with similar sequence specificities.

We next examined the effect of heat shock on the binding activity. Whereas HeLa cells heat shocked to 45 °C for 30 min contained readily detectable levels of a specific HSE-binding protein (Fig. 1*b*), no activity could be detected in control cells. This inducibility supports the idea that the HSE-binding activity is involved in the regulation of heat shock genes. Induction probably involves modification of an existing protein, because it is not blocked by cycloheximide (data not shown). Similar results have recently been described by Kingston *et al.*[16].

Different results were obtained when control and heat-shocked yeast cells were compared. Cells grown at 23 °C contained high levels of binding activity, and its amount did not change when the cells were heat shocked for 20 min at temperatures ranging from 34 °C to 39 °C (Fig. 3*a*). Moreover, the

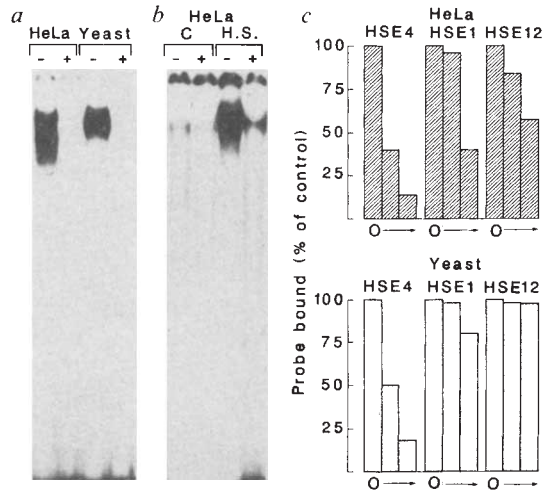

Fig. 1. HSE-binding proteins in HeLa cells and yeast. *a*, Gel electrophoresis of complexes formed with a dimeric HSE probe[12] and extracts from either heat-shocked HeLa cells or yeast. Assays were performed in the absence (−) or presence (+) of a 100-fold molar excess of unlabelled probe. Free probe has migrated off the bottom of the gel. *b*, Inducibility of binding activity in HeLa cells. Extracts[22] (containing twice as much protein as in *a*) from control (C) and heat-shocked cells (H.S.; 45 min at 45 °C) were assayed with (+) or without (−) unlabelled competitor. *c*, Specificity of binding. Extracts from HeLa cells (top) and yeast (bottom) were incubated with labelled dimeric HSE probe (containing the sequence CTAGAAGCTTCTAGAAGCTTCTAG, the HSE consensus being CNNGAANNTTCNNG), in the absence (0) or presence of increasing quantities (arrows) of unlabelled competitor DNA. The competitor contained either a single 8/8 match to the consensus HSE (HSE1, CTAGAAGCTTCTAG), four overlapping copies of this sequence (HSE4, CTAG(AAGCTTCTAG)$_4$) or two overlapping copies of a 6/8 match to the consensus (HSE12, CTAGAGATCTCTAGAGATCTCTAG)[12]. The amount of bound probe was quantified by counting appropriate regions excized from gels. For HeLa extracts, successive bars correspond to a competitor: probe molar ratio of 0, 4 and 20; for yeast extracts the ratios were 0, 10 and 50.

Methods. Yeast extracts were prepared from the multiply protease-deficient strain BJ2168 (*leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*; a gift from D. Shore) and fractionated on heparin-agarose and calf thymus DNA-sepharose as described elsewhere[12]. Binding reactions (20 μl) contained up to 40 μg of extract protein, 0.5 ng ^{32}P-end-labelled 62-bp probe, 1 μg supercoiled pUC19 DNA, 1 μg poly[d(I-C)], 20 mM HEPES *p*H 7.9, 1 mM $MgCl_2$, 60 mM KCl, 12% glycerol, 0.1% NP40 and 1 mM dithiothreitol. They were incubated for 30 min at room temperature and analysed on 4% polyacrylamide gels run in 22.5 mM Tris-borate, 0.6 mM EDTA. For competition experiments, the total amount of plasmid DNA was kept constant.

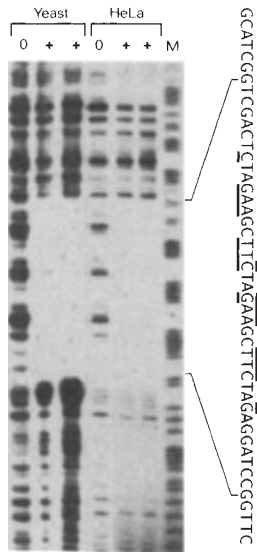

Fig. 2. Footprinting with yeast and HeLa extracts. The probe was derived from the plasmid pTKS2 (ref. 7). It was mixed with poly[d(I-C)] as carrier, and digested with DNase I after incubation without (0) or with (+) the yeast and HeLa extracts used in Fig. 1. M, marker track (the probe partially cleaved at purine residues). The DNA sequence is shown at the right, with the two overlapping HSE consensus sequences indicated by lines.

stability of DNA-protein complexes formed with extracts of control and heat-shocked cells, as estimated from their dissociation rates in the presence of excess unlabelled probe, was identical. With a monomeric HSE probe, the half-life of the complexes was ~20 min (Fig. 3*c*); complexes formed with a dimeric HSE in both control and heat-shocked extracts were more stable, and no dissociation was detected within two hours.

These results could be explained if our conditions for growing and harvesting the cells resulted in accidental heat-shock. Alternatively, the synthetic HSE might have some special property that causes it to be constitutively active. To test these possibilities, we introduced into yeast an expression plasmid in which the dimeric HSE sequence used in our binding assays lies upstream of a *CYC1-lacZ* fusion gene (this construct lacks the normal *CYC1* upstream activating sequences). The activity of the promoter was then monitored by β-galactosidase assay. Very little activity was detected in cells grown at 23 °C but after heat shock for 20 min at temperatures between 34 °C and 39 °C there was a clear temperature-dependent induction of activity (Fig. 3*a*). When cells grown at 23 °C were centrifuged and resuspended as though for extract preparation, and then returned to growth medium, there was no induction of β-galactosidase activity (Fig. 3*b*). These results show that the synthetic HSE is functional in yeast and that despite its ability to form very stable protein-DNA complexes *in vitro*, it does not activate transcription constitutively. Furthermore, the presence of HSE-binding activity in control cells cannot be explained by accidental heat shock.

We conclude that the yeast heat-shock factor can bind to DNA whether or not cells are stressed. This implies that its ability to activate transcription is regulated independently of its ability to bind DNA. To see whether we could detect any heat-induced alterations in the factor, we examined the electrophoretic mobility of protein-HSE complexes formed in control and heat-shock extracts. Figure 4 shows examples of gels run for longer than normal, to accentuate possible differences. Two bands are visible in each lane, a major and a minor, more slowly migrating one. Extracts made from cells incubated at 39 °C for 20 min yielded complexes whose mobility was clearly reduced relative to those from control extracts (compare lanes 13 and 14). This effect could not be produced by heat shock of the control extract *in vitro* (lane 6), which rules out simple denaturation of the binding protein as its cause.

Yeast cells respond very rapidly to heat shock, but the response is transient, lasting no more than an hour[17]. Similarly, the mobility shift was detectable within three minutes of exposure to 39 °C (Fig. 4*a*) and was reversed within 30 min of recovery at 23 °C (Fig. 4*c*). The change in mobility was clearly progressive, during both induction of the response and recovery. The temperature required to induce a change in the protein-HSE complexes (Fig. 4*b*) also correlated well with the activation of heat shock transcription (Fig. 3*a*): the greatest change in mobility was observed at 39 °C, a partial effect at 36 °C, and a small effect at 33 °C. These results indicate that the changes detected by the gel assay are intimately connected with heat-shock gene activation.

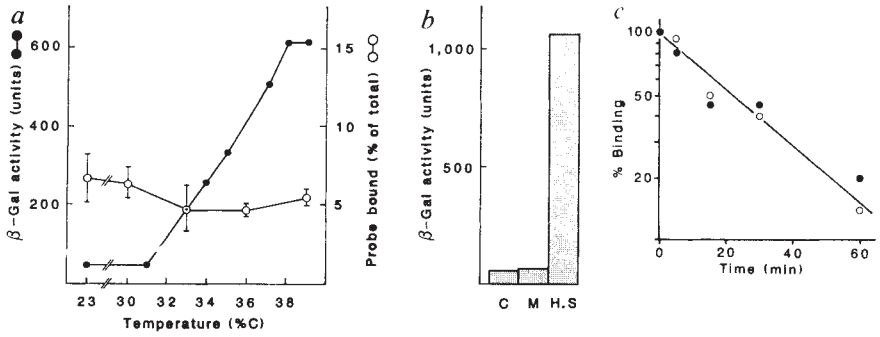

Fig. 3. Detection of yeast HSE-binding activity does not require induction of the heat shock response. *a*, The β-galactosidase and HSE-binding activity in cells transformed with an HSE-*cyc1-lacZ* fusion gene. Cells were heat-shocked for 20 min at the indicated temperatures, and allowed to recover for 1 h at 23 °C. The β-galactosidase activity (●) was determined as described previously[23]. Similarly treated cultures were harvested immediately after heat shock and assayed for HSE-binding activity. Assays were performed on duplicate cultures; the mean and range are shown (○). *b*, Transformed yeast cells were grown at 23 °C and β-galactosidase activities determined as in *a*. Values shown are for control cells (C), cells heat shocked at 39 °C (H.S.), and cells grown at 23 °C, harvested as though for determination of HSE-binding activity (see below), but resuspended in medium instead of being frozen in liquid N_2. After further incubation for 1 h at 23 °C, these mock-harvested cells (M) were assayed for β-galactosidase activity by the standard protocol. *c*, Stability of HSE-protein complexes. Binding reactions containing monomeric HSE probe and either heat shocked (●) or control (○) yeast extracts were incubated for 15 min before addition of a large excess of unlabelled competitor DNA (tetrameric HSE). After the indicated times, remaining complexes were assayed by gel electrophoresis.

Methods. The dimeric HSE sequence was inserted upstream of the TATA box of a disabled *cyc1-lacZ* fusion gene in the vector pLGΔ-178 (which contains the URA3 gene)[24], and the plasmid introduced into the strain BJ2168. Transformed cells were grown to early log phase in selective medium at 23 °C before assay. For determination of HSE-binding activity, cells were harvested by centrifugation, resuspended in 200 mM Tris-HCl *p*H 8.0, 10 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 0.5 mM TPCK, 0.025 mM TLCK, 2 μg ml^{-1} pepstatin A, rapidly frozen in liquid nitrogen, and ground in a mortar whilst still frozen. After thawing and centrifugation in a microfuge to remove debris, the extracts were assayed as in Fig. 1.

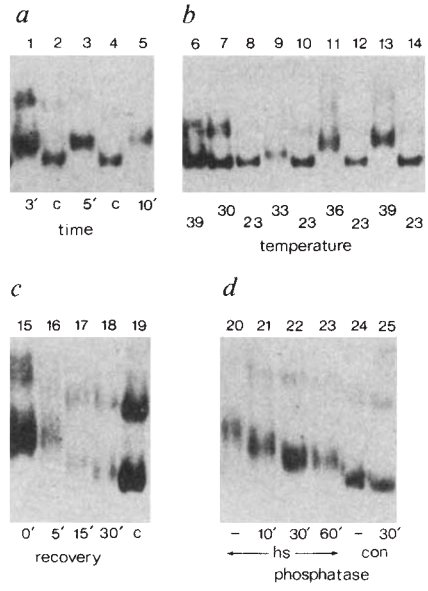

Fig. 4. Effect of heat shock on the mobility of protein-HSE complexes from yeast. Assays were performed as in Fig. 1, except that gels were run for 4 h (*a* and *b*) or 6 h (*c* and *d*) rather than 1 h. *a*, Time course of induction. Cells were grown at 23 °C, then incubated at 39 °C for the indicated number of minutes before harvesting. Lanes 2 and 4 are unshocked controls. *b*, Effect of heat shock temperature. Cells were heat shocked for 20 min at the indicated temperature (lanes 9, 11, 13) grown at 30 °C (lane 7) or left at 23 °C (lanes 8, 10, 12, 14). Lane 6 contains control extract that was heated to 39 °C for 20 min before assay. *c*, Recovery *in vivo*. Cells were heat shocked for 20 min at 39 °C and allowed to recover in an orbital shaker at 23 °C for the indicated times before harvesting; lane 19 shows an unshocked control. *d*, phosphatase treatment. Extracts containing 120 μg of total protein from heat shocked cells (hs; 20 min at 39 °C) or control cells (con) were incubated at 37 °C for 30 min without phosphatase (lanes 20, 24) or for the indicated times with 20 units of calf intestinal phosphatase (Boehringer Mannheim) before assay.

What causes the change in mobility? A likely candidate is phosphorylation of the binding protein, and to test this we added calf intestinal phosphatase to the extracts. This had no effect on the complexes from control extracts (Fig. 4*d*, lanes 24 and 25), but progressively increased the mobility of the complexes formed in heat-shocked extracts (lanes 20–23). In the experiment shown in Fig. 4*d*, a residual mobility difference remained even after prolonged phosphatase treatment. This was frequently the case, although with at least one extract an apparently complete recovery was observed. A likely explanation of these results is that heat shock causes phosphorylation of the HSE-binding protein at multiple sites, and that some phosphates are removed from the protein very inefficiently. Nevertheless, we cannot rule out the possibility that an additional form of modification also occurs.

We have recently purified a 150K HSE-binding protein from unshocked yeast, and shown that it accounts for all the complexes seen when crude extracts are analysed by gel electrophoresis[12]. Presumably, therefore, it is this protein that is phosphorylated upon heat shock; direct proof of this awaits isolation of the protein from heat-shocked cells.

The conclusion from this work is that the mechanism of heat-shock gene activation differs between yeast and HeLa cells, even though the regulatory sequences are highly conserved. In HeLa cells, the affinity of the heat-shock factor for DNA is increased by heat shock, and binding presumably activates transcription. In yeast, the affinity of the factor for DNA does not change, and it must therefore be the nature of its interaction with other components of the transcriptional machinery that is altered. This does not exclude the possibility that the factors are modified by a common mechanism. Our results indicate that the yeast factor is modified by phosphorylation. So far, however, we have not been able to observe any effect of phosphatase on the HeLa cell factor.

In several instances, activation of transcription has been correlated with a change in the binding of putative transcription factors to DNA[18,19]. In other cases, this correlation does not hold—for example, a protein binding the c-*fos* serum response element can be detected in both starved and serum-fed cells, although its activity is presumably modulated *in vivo*[20,21]. The yeast heat-shock response may provide a precedent for the regulation of gene activity by phosphorylation of a constitutively bound factor.

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Note added in proof: We have recently purified the HSE-binding protein from heat-shocked yeast and shown that it is phosphorylated.

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A bacterial calcium-binding protein homologous to calmodulin

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Many of the effects of calcium ions in eukaryotic cells are mediated by calcium-binding regulatory proteins such as calmodulin, in which each calcium-binding site has a distinctive helix–loop–helix conformation termed the EF hand[1,2]. Protein S from the spore coat of the Gram-negative bacterium *Myxococcus xanthus* has been shown to resemble calmodulin in its internally-duplicated structure and ability to bind calcium[3]. However, it has a β-sheet secondary structure[4,5] rather than the helix–loop–helix arrangement of the eukaryotic proteins. We have determined the complete amino-acid sequence of a calcium-binding protein[6] from the Gram-positive bacterium "*Streptomyces erythraeus*" by cloning and sequencing the corresponding gene. It contains four EF-hand motifs bearing remarkable sequence similarity to the calcium-binding sites in calmodulin. This implies that the EF-hand superfamily may have evolved from ancient proteins present in prokaryotes.

Calmodulin is the most prominent member of a family of small, acidic calcium-binding proteins, found in eukaryotic cells, through which calcium regulates a variety of cellular processes. The sequence similarity between these proteins is fairly extensive and is centred on the calcium-binding sites, of which there are four in calmodulin. Each calcium-binding site consists of a sequence of about 30 amino acids in which α-helical segments flank a 12-residue calcium-binding loop. Kretsinger has termed this structural motif the EF hand[1,2]. Calmodulin has an established role in the activation of a number of enzymes involved in cellular regulation (see refs 7-9 for reviews). In contrast, the role of calcium-binding proteins in prokaryotes is obscure, although calmodulin-like activity has been reported in *Escherichia coli*[10] and *Bacillus subtilis*[11]. When a small, acidic protein from the erythromycin-producing bacterium "*Streptomyces erythraeus*" was found to contain an EF hand motif and was shown to bind calcium[6], it led us to examine the primary structure of this protein in more detail.

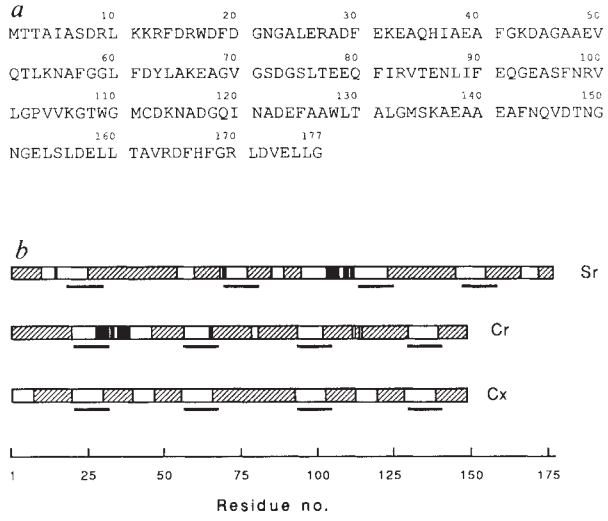

Fig. 1 "*S. erythraeus*"[26] calcium-binding protein: amino-acid sequence deduced from the sequence of the structural gene, and predicted secondary structure. *a*, The four potential calcium-binding loops span residues 20-31, 69-80, 113-124 and 147-158 respectively. *b*, The Robson[14] algorithm was used to predict the secondary structure of "*S. erythraeus*" calcium-binding protein (Sr) and human calmodulin (Cr). The secondary structure of human calmodulin as deduced from X-ray crystallography[15] is also shown (Cx). Hatched boxes, α-helical segments; filled boxes, β-sheet; unfilled boxes, β-turn or random coil conformations. Potential calcium-binding loops are underlined.
Methods. The structural gene for the calcium-binding protein from "*S. erythraeus*"[26] NRRL 2338 was detected, in chromosomal DNA cut with *Kpn*I, using oligonucleotide probes designed from a knowledge of part of the amino-acid sequence, and cloned into the multicopy vector pUC18 in *E. coli*. A 0.8-kilobase (kb) DNA fragment containing the entire structural gene was sequenced using the Sanger dideoxy chain termination method[27]. Details of the cloning, the complete DNA sequence of this fragment and criteria for identifying the reading frame will be described elsewhere.

We have isolated and sequenced the structural gene for the calcium-binding protein from "*S. erythraeus*" and deduced the complete amino-acid sequence of the protein (Fig. 1*a*). The protein contains 177 amino acids (relative molecular mass (M_r) 20,090), compared to the 148 amino acids in human calmodulin[12] (M_r 16,723). Like calmodulin, the "*S erythraeus*" protein contains a high percentage of negatively charged amino acids, but the overall amino-acid composition is distinctly different.

The amino-acid sequences of the "*S. erythraeus*" calcium-binding protein and of human calmodulin were compared using the Diagon algorithm of Staden[13], in which two protein sequences are displayed along the x and y axes, respectively, and scored for similarity. Several regions of similarity were revealed (Fig. 2), and these corresponded exactly to the positions of the potential calcium-binding sites in both proteins. The pattern is not perfectly regular, because the calcium-binding sites in the bacterial protein are not as evenly-spaced along the polypeptide chain as they are in calmodulin. Calcium-binding site II in the "*S. erythraeus*" protein (residues 113-124) is only revealed, at this level of stringency, by alignment with one out of the four calcium-binding sites in calmodulin. The amino-acid sequence in the N-terminal part of the presumed calcium-binding loop