The EMBO Journal vol.11 no.5 pp.1857 - 1866, 1992

Repression of the H5 histone gene by a factor from erythrocytes that binds to the region of transcription initiation

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Communicated by M.Beato

Expression of histone H5, like that of other erythrocyte specific proteins, declines during the latter stages of erythroid maturation because of a decrease in the rate of gene transcription. Here, we report the isolation of cIBR (chicken initiation binding repressor), a 75 kDa DNA binding glycoprotein from mature chicken erythrocytes that recognizes sequences spanning the transcription start sites of the *H5* gene. cIBR was found to repress transcription from the *H5* promoter *in vitro* and this effect could be relieved by mutations that lowered the affinity of the factor for its cognate sequence. cIBR inhibited transcription by interfering with assembly of the initiation complex, but it did not affect transcription from pre-assembled complexes. Consistent with this, binding of bacterially expressed human TFIID to the TATA element prevented subsequent binding of cIBR, although the opposite was not true. This, and the fact that cIBR had no effect when bound in a location upstream from the promoter, suggests that binding of cIBR to the start site region causes repression by direct interference with general transcription factors other than TFIID, possibly TFIIB. cIBR was found in mature and relatively late erythrocytes but not in early erythroid cells which actively transcribe the *H5* gene; the transcriptionally active cells contain instead cIBF (chicken initiation binding factor). Purified cIBF is a non-glycosylated 68–70 kDa DNA binding protein(s) which also recognizes the region of transcription initiation of the *H5* gene.
Key words: cIBF/cIBR/erythroid/histone *H5* gene/repressor/TFIID/transcription initiation

Introduction

H5 is an erythrocyte specific linker histone, the expression of which is regulated in a stage specific manner during maturation of avian red blood cells (Affolter *et al.*, 1987 and references therein). Although *H5* is expressed in early HD3 cells [avian erythroblastosis virus transformed adult CFU(E)], its level increases significantly during the transition to erythroblasts. This is partly due to the transcriptional activation of the gene by any of three stage specific enhancers (S.Rousseau, J.Renaud, M.Affolter and A.Ruiz-Carrillo, in preparation, see Trainor *et al.*, 1987; Rousseau *et al.*, 1989). The higher *H5* gene activity is maintained during subsequent stages of maturation (Affolter *et al.*, 1987) until the cells approach maturity. Little transcriptional activity remains in the late polychromatic and mature erythrocytes (Ruiz-Carrillo *et al.*, 1974).

The terminally differentiated erythrocyte represents a paradigm of a genetically inactive somatic cell (Lucas and Jamroz, 1961). The general chromatin inactivity of the erythrocyte is thought to be partly caused by histone H5 and we have recently shown that H5 can suppress chromatin activity in non-erythroid cells when expressed at levels comparable to those of mature erythrocytes (Sun *et al.*, 1989; see also Bergman *et al.*, 1988). Although the higher condensation or stability of chromatin containing H5 relative to H1 may contribute to nuclear inactivation (Sun *et al.*, 1990), it is clear that transcription of all active genes is not affected to the same degree by the replacement of H1 by H5 (Affolter *et al.*, 1987; Sun *et al.*, 1989). Genes transcribed in the maturing erythrocyte are likely to be progressively silenced by a change in the balance between positive and negative effectors.

Selective transcriptional repression plays an important role in the regulation of gene expression, and the mechanisms of repression are likely to be as varied as those of activation (for reviews see Levine and Manley, 1989; Renkawitz, 1990). Even though repression can be achieved by interference of any step of gene activation, a directly acting repressor would alter the interaction of RNA polymerase or general transcription factors with a promoter. This mechanism is commonly observed in prokaryotic gene regulation and probably accounts for the negative effect of SV40 T antigen on its own promoter (Rio and Tjian, 1983). Blocking of TFIID binding to the TATA element has also been proposed as an example of dominant negative regulation during development (Ohkuma *et al.*, 1990) and *HIV-1* transcription (Kato *et al.*, 1991). This type of negative regulation appears as highly efficient to suppress basal as well as induced levels of promoter activity. However, the number of eukaryotic genes that have been shown to use this mechanism is so far very limited.

We report here the characterization of cIBR, a novel transcription factor from mature erythrocytes that acts as a repressor of *H5* gene transcription *in vitro* and explore its mechanism of action. cIBR was not detected in induced HD3 cells that actively transcribe the *H5* gene and therefore its appearance parallels the decline in activity of the *H5* gene. We also report the characterization of a DNA binding activity from HD3 cells (cIBF) that recognizes the same DNA region as cIBR. Although cIBR and cIBF could be related proteins, their transcriptional effects and some of their properties are clearly distinguishable.

Results

Recognition of the transcription initiation region of the histone H5 gene by nuclear proteins from erythroid cells

The −95 to +72† sequences of the *H5* gene are sufficient for transcription in non-induced HD3 cells. The functional elements of this promoter, other than the TATA element, include a high affinity Sp1 binding site (−88 to −81) and a H4TFII binding site (−55 to −38) (Rousseau *et al.*, 1989). To characterize further the factors that control promoter activity, we performed DNase I protection assays with nuclear proteins from mature erythrocytes (ME) and HD3 cells. These experiments revealed several footprints, including the transcription initiation region (Figure 1A, lanes 3 and 4; Figure 1B, lanes 3−6). Interestingly, the sequences protected by proteins from ME and induced or non-induced HD3 cells are different and the interaction appears to be specific as shown by competition with homologous or heterologous oligonucleotides (Figure 1A, lanes 5, 8 and 6, 7, respectively). We focused our attention on the characterization of these activities because of the particular importance of the transcription initiation region. Although there are examples of DNA binding proteins that recognize the start site of other genes (Rio and Tjian, 1983; Muller, 1987; Ayer and Dynan, 1988; Means and Farnham, 1990, Beaupain *et al.*, 1990), the relevant sequence of the *H5* gene suggested that the activities detected had not been previously characterized.

The protein−DNA complexes were characterized by electrophoretic gel retardation assays using a probe (TIS) spanning the transcription initiation site (−20 to +14). Figure 2A shows that a complex of identical mobility was observed with nuclear proteins from ME and HD3 cells. The specificity of the complex was tested by competition with increasing amounts of wild type and mutant TIS oligonucleotides (Figure 2B, the mutant contained several transversions of the nucleotides contacted by the binding proteins, see Figure 5E). Complex formation was increasingly inhibited by wild type oligonucleotide (Figure 2A, lanes 3, 4), but not by mutant oligonucleotide (lanes 5, 6). Complex formation was also resistant to competition by oligonucleotides containing the recognition sequences for *H5* CACCC factor, *H5* Sp1, *H4* TFII, *H5* GATA-1 of the 3′ enhancer (Rousseau *et al.*, 1989), human porphobilinogen deaminase NF-E2 (Mignotte *et al.*, 1989), SV40 AP1 (Angel *et al.*, 1987; Lee *et al.*, 1987) and cloned $(dG)_n(dC)_n$ tracts [n = 17, 29], (data not shown). These results indicate that the complexes are specific.

Purification of cIBR from mature erythrocytes

The DNA binding protein from ME (hereafter referred to as cIBR) was purified by wheat germ agglutinin−Sepharose chromatography (WGA), since pilot tests had indicated that it was glycosylated (details of the purification are given in Materials and methods). More than 99% of the proteins from the nuclear extract eluted in the flow through, whereas virtually all of the cIBR was retained and was eluted with

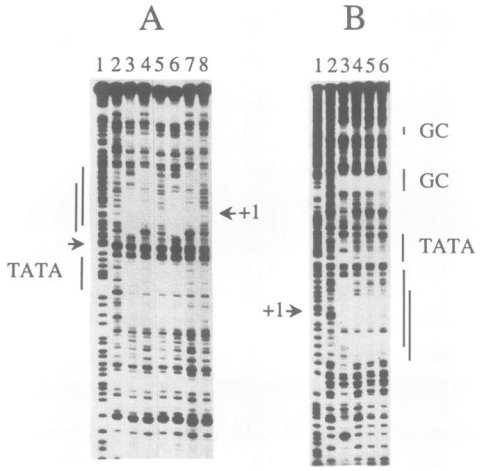

Fig. 1. Interaction of nuclear proteins from erythroid cells with the *H5* promoter. DNase I footprinting reactions were performed with a probe (−307 to +72) labelled at the 5′ end of the coding (**A**) or the non-coding strand (**B**). (A) Lane 1, chemical G+A sequencing reaction; lane 2, no protein; lanes 3, 5 and 6, mature erythrocytes (25 μg); lanes 4, 7 and 8, non-induced HD3 (40 μg). Lanes 5, 8 and 6, 7, respectively, a 100 molar excess of TIS oligonucleotide or of an oligonucleotide containing the recognition sequence of NFI was included during the binding reaction. (B) Lane 1, G+A chemical sequencing reaction; lane 2, no protein; lane 3, mature erythrocytes (25 μg); lane 4, induced HD3 (10 μg); lane 5, non-induced HD3 (40 μg); lane 6, non-inducible HD3−41/2 (45 μg).

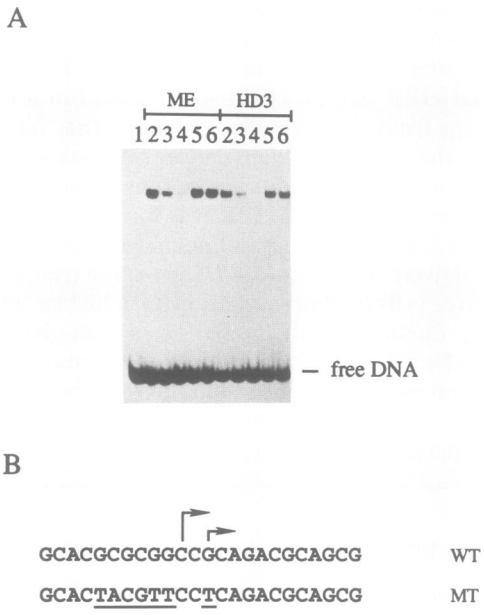

Fig. 2. (**A**) Specificity of protein binding to the transcription initiation region of the *H5* gene. The electrophoretic mobility shift assay was performed with 5′ end-labelled TIS probe and nuclear extracts from mature erythrocytes (ME, 15 μg) and non-induced HD3 cells (7.5 μg). Lane 1, no protein; lanes 2, no competitor; lanes 3 and 4, 20 and 80 molar excess of wild type TIS oligonucleotide; lanes 5 and 6, 20 and 80 molar excess of mutant TIS oligonucleotide. (**B**) Sequences of the wild type (WT) and mutant (MT) TIS oligonucleotides used in the competition.

†The 5′ end of *H5* mRNA was mapped by us (Ruiz-Carrillo *et al.*, 1983) and Wigley *et al.* (1985) at different positions. This has been re-examined by reverse transcription of *H5* mRNA from immature erythrocytes and sequencing of the extended product. The data clearly indicated that the major 5′ end of the mRNA maps 5 nt downstream from our previous determination and 3 nt upstream from that of Wigley *et al.* (1985), and confirmed that the *H5* mRNA leader contains the sequence GGCGGCAGC which is absent in the cloned alleles of the gene (Krieg *et al.*, 1983; Ruiz-Carrillo *et al.*, 1983; Figure 6C of this paper). The transcription start site (+1) used in the present work takes these results into account.

N-acetylglucosamine (Figure 3A, lane 1; 3B, lanes 1, 2; 3C, lane 3). In fact, cIBR had the same behaviour as Sp1, known to contain O-linked *N*-acetylglucosamine groups (Jackson and Tjian, 1988) which interact with two GC boxes upstream of the *H5* promoter (Figure 1B and Figure 3C, lane 3; and unpublished observations). When the cIBR fraction was applied to a DNA affinity resin containing concatemers of TIS oligonucleotide, most of the protein, including Sp1, eluted in the flow through (Figure 3C, lane 4), whereas cIBR was retained. The column was washed stepwise with 0.25, 0.5 and 1.0 M KCl (Figure 3A, lanes 2–4) and the bulk of cIBR was found in the 0.5 M KCl fraction (Figure 3B, lanes 3 and 4). A second round of affinity chromatography yielded a fraction containing a 75 kDa doublet (Figure 3A, lane 5) that footprinted on the transcription initiation region (Figure 3C, lane 5) and was competed with an excess of the homologous sequence (Figure 3C, lane 6). Purified cIBR was able to bind to a WGA column and was eluted with *N*-acetylglucosamine, demonstrating that retention in the first WGA chromatography was due to its glycosylation rather than to indirect binding.

To demonstrate that the proteins comprising the 75 kDa doublet had cIBR activity, the 0.5 M KCl eluate from the second DNA affinity chromatography was further fractionated by preparative gel electrophoresis. Three slices of the gel corresponding to 90–130 kDa, 65–80 kDa and 40–45 kDa were eluted, and the extracted material was renatured and used in gel retardation assays. Figure 3B shows that cIBR was present in the 65–80 kDa fraction (lanes 5 and 6), whereas no activity was found in the other gel slices (lanes 7 and 8).

The heterogeneity of cIBR could reflect the presence of different proteins, post-synthetic modifications or proteolytic degradation. Despite the fact that protease inhibitors were used during the isolation, we attribute the heterogeneity to degradation, since different preparations of cIBR show different proportions of the two electrophoretic species that co-purify in additional rounds of affinity chromatography. The degradation hypothesis is also supported by the relatively simple pattern of peptides obtained by chemical cleavage with a tryptophan specific reagent (data not shown).

Purification of cIBF from HD3 cells

The identical electrophoretic mobility of the complex made by the ME and HD3 nuclear proteins (Figure 2A) initially suggested that cIBR was also present in HD3 cells.

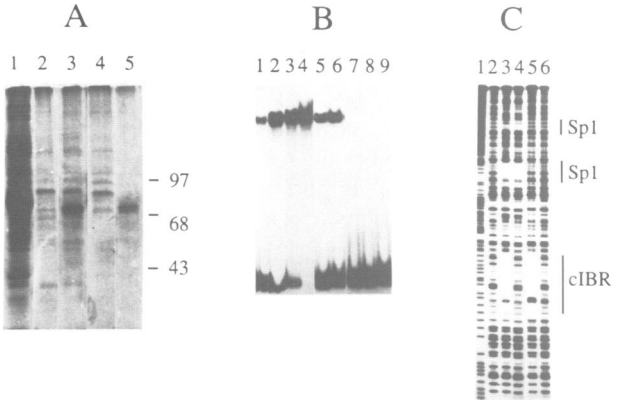

Fig. 3. Purification of cIBR from mature erythrocytes. (**A**) Selected samples from fractions analysed by electrophoresis. Lane 1, proteins eluted with 0.3 M *N*-acetylglucosamine from a wheat germ agglutinin (WGA) column; lanes 2–4, 0.25, 0.5 and 1.0 M KCl washes of a TIS oligonucleotide resin; lane 5, 0.5 M KCl wash of a second round of DNA affinity chromatography. The mobility of protein standards (in kDa) is indicated. (**B**) Electrophoretic mobility shift assay with cIBR fractions. Lanes 1 and 2, 12.5 and 25 ng of proteins eluted from the WGA resin with 0.3 M *N*-acetylglucosamine; lanes 3 and 4, 5 and 10 ng of the 0.5 M KCl fraction from the second DNA affinity chromatography; lanes 5 and 6, 5 and 10 μl (from a total of 100 μl) of renatured material eluted from the 65–80 kDa region of a preparative gel electrophoresis of purified cIBR; lanes 7 and 8, 10 μl each of renatured material eluted from the 90–130 and 40–45 kDa regions of the same gel; lane 9, no protein. (**C**) DNase I footprints of cIBR fractions. Reactions were performed with a probe (−307 to +72) labelled at the 5′ end of the non-coding strand. Lane 1, G+A chemical sequencing reaction; lane 2, no protein; lane 3, 250 ng of proteins eluted from the WGA resin with 0.3 M *N*-acetylglucosamine; lane 4, 200 ng of the flow through fraction from the first DNA affinity chromatography; lane 5, 20 ng of the 0.5 M KCl fraction from the second DNA affinity chromatography; lane 6, as in 5 except that a 400-fold molar excess of the TIS oligonucleotide was included during the binding reaction.

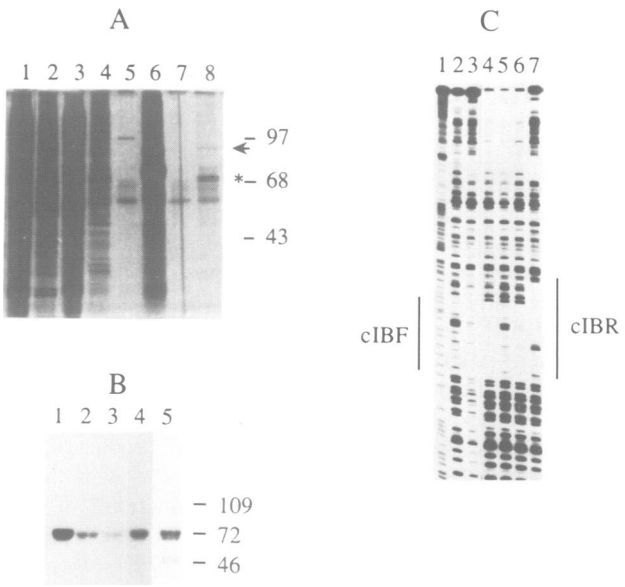

Fig. 4. Purification of cIBF. A nuclear extract from HD3 cells was fractionated by chromatography in WGA–agarose, heparin–agarose and two rounds of DNA affinity chromatography. (**A**) Silver staining of selected protein fractions. Lane 1, heparin–agarose; lanes 2–5, first DNA affinity chromatography, flow through (2), 0.25 M KCl wash (3), 0.5 M KCl wash (4), 1.0 M KCl wash (5); lanes 6–8, second DNA affinity chromatography, flow through (6), 0.25 M KCl wash (7), 0.5 M KCl wash (8). The mobility of protein standards (in kDa) is indicated. (*) shows the position of cIBF, and the arrow that of a 90 kDa contaminant. The lower molecular weight species in lanes 5, 7 and 8 were also present in lanes containing sample buffer alone and are artifacts. (**B**) UV crosslinking of cIBF. Autoradiography of proteins resolved by SDS–PAGE. 2.5 μg of the heparin–agarose fraction was reacted with a radioactive BrdU substituted 49 bp cIBF probe in the presence of 100 ng of poly(dAdT) and the indicated competitors. Lane 1, no competitor; lanes 2, 3, 5 and 10 molar excess of TIS oligonucleotide; lanes 4, 10 molar excess of an AP1 oligonucleotide; lane 5, no competitor (from a different experiment). (**C**) Selected DNase I footprints with cIBF fractions. Reactions performed with a −95 to +72 *H5* promoter probe labelled at the 5′ end of the non-coding strand. Lane 1, chemical G+A sequencing reaction; lane 2, no protein; lane 3, 25 μg of the HD3 nuclear extract; lane 4, 25 μg of the WGA chromatography flow through fraction; lane 5, 25 ng of the 0.25 M KCl fraction (second DNA affinity chromatography); lane 6, 15 ng of the 0.5 M KCl fraction (second DNA affinity chromatography); lane 7, 250 ng of cIBR purified by WGA chromatography.

Surprisingly, however, when a nuclear HD3 extract was applied to a WGA resin, the binding activity (hereafter referred to as cIBF) was recovered in the flow through and not in the WGA-bound fraction (Figure 4C, lane 4). This behaviour was not due to saturation of the resin as judged from the retention of Sp1 (data not shown). Therefore, cIBF was first enriched by heparin–agarose chromatography before purification by two rounds of DNA affinity chromatography using immobilized TIS oligonucleotide (Figure 4, A and C; details of the purification are given in Materials and methods). The 0.5 M KCl wash of the second DNA affinity chromatography yielded a fraction composed of two main proteins of 68 and 70 kDa and a minor of ~90 kDa (Figure 4A, lane 8). The footprint obtained with purified cIBF (Figure 4C, lane 6) indicates that the region protected is clearly different from that protected by cIBR (cf. lanes 6 and 7), although it overlaps with it, extending farther into the downstream sequences (see Figure 5E for a summary of cIBR and cIBF footprints). Purified cIBF was not retained when applied to a WGA resin, confirming that it is not modified by *N*-acetylglucosamine residues.

To ascertain that the activity of cIBF corresponded to the 68–70 kDa proteins, we attempted, albeit unsuccessfully, to fractionate the 0.5 M KCl affinity fraction by SDS–PAGE. Since purified cIBF proved to be highly unstable, we resorted to UV crosslinking. A DNA probe containing the cIBR/cIBF binding site was prepared by incorporating bromodeoxyuridine and radioactive deoxycytidine into the non-coding strand (Chodosh *et al.*, 1986). The internally labelled probe was incubated with partially purified cIBF (heparin fraction) under conditions of complex formation. After binding, the samples were irradiated with UV light and extensively digested with DNaseI and micrococcal nuclease. Electrophoresis of the crosslinked proteins indicated the presence of a major radioactive product of ~72 kDa (Figure 4B, lane 1), a size compatible with that of purified cIBF, taking into account an increase in mass contributed by a crosslinked nuclease resistant oligonucleotide fragment. The specificity of the adduct was assessed by competition with homologous and heterologous oligonucleotides. Figure 4B (lanes 2 and 3) shows that the signal was significantly decreased when a five and 10 molar excess of non-labelled TIS oligonucleotide was included during binding, a reduction higher than that observed by competition with an unrelated oligonucleotide (containing a SV40 AP1 site, Figure 4B, lane 4). Although the results shown in Figure 4B (lanes 1–4) do not indicate the presence of a doublet, we believe that this is due to a lack of resolution in this particular electrophoresis, since a doublet can be seen in several other crosslinking experiments (Figure 4B, lane 5). We conclude therefore that cIBF corresponds to the 68–70 kDa species purified by DNA affinity chromatography (Figure 4A, lane 8). At present it is not known whether cIBF is a heterotypic complex of the 68 and 70 kDa proteins or whether the heterogeneity is due to degradation or differential postsynthetic modification. In any event, cIBF is slightly smaller than cIBR, a difference that was confirmed by running the purified proteins side by side in the same gel (data not shown).

cIBR and cIBF show the same methylation interference pattern

To examine the DNA contacts established by cIBR and cIBF in greater detail, we performed binding interference assays with a TIS probe partially methylated with dimethylsulfate. Comparison of the methylation patterns of free and factor-complexed DNA indicates that methylation of the same guanine residues affects binding of both factors (Figure 5, A, B, and E), suggesting that they recognize similar features of the major groove of DNA. The region of interference spans from −6 to +4 and includes sequences with dyad symmetry at the major site of transcription initiation (Figure 5E). Results of gel filtration experiments indicated that cIBR behaves in solution as a 150 kDa species (FPLC

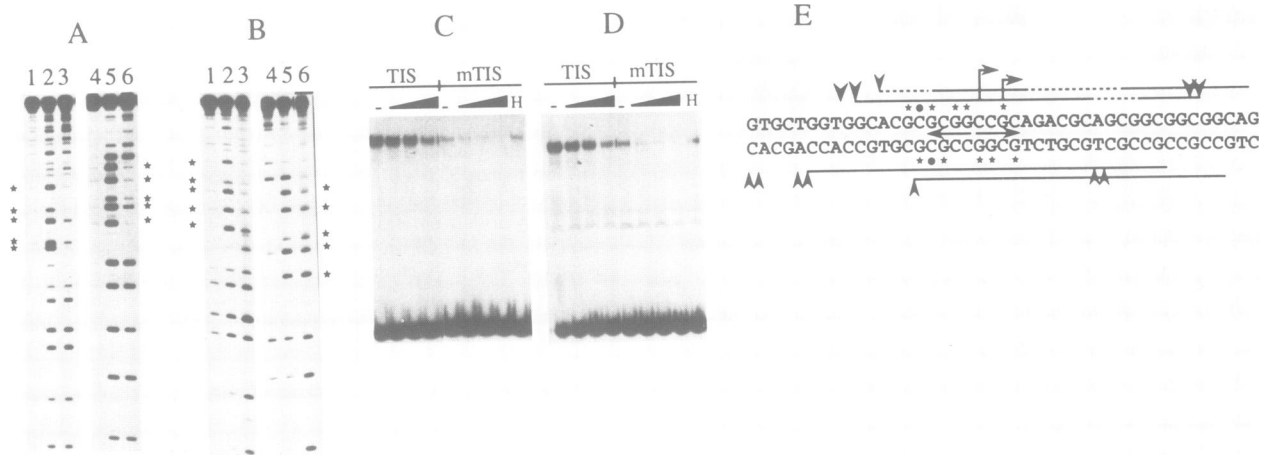

Fig. 5. Methylation interference assays. (**A** and **B**) Partially DMS-methylated TIS oligonucleotide was labelled at the 5′ end of the coding (lanes 1–3) or the non-coding strand (lanes 4–6) and incubated with partially purified cIBR (A) or cIBF (B). Free and factor-bound DNA was purified and hydrolyzed with piperidine. (A) Lanes 1 and 4, non-methylated oligonucleotide; lanes 2 and 5, free DNA; lanes 3 and 6, cIBR-bound DNA. (B) Lanes 1 and 4, cIBF-bound DNA; lanes 2 and 5, free DNA; lanes 3 and 6, G+A sequence. The anomalous mobility of the ladders in lanes 1–3 of panel (A) is due to compression. (**C** and **D**) 5′ end labelled TIS oligonucleotide was methylated at the −5 cytidine with *Hha*I methylase, or mock methylated. Equal amounts of methylated (mTIS) and non-methylated (TIS) probes were used in gel retardation assays with purified cIBR (C) and cIBF (D). Lanes: (−), no competitor oligonucleotide; competition with 5, 10 and 30 molar excess of TIS oligonucleotide during binding; H, probe digested with *Hha*I before binding. (**E**) Summary of DNase I protection and methylation interference results. The footprints (horizontal lines) were obtained with the affinity purified cIBR (inner) and cIBF (outer). The extent of protection is indicated by solid (stronger) and dashed (weaker) lines. DNase I hypersensitive sites are shown by arrowheads. (*) and (●) indicate G and C methylation interference, respectively. A palindrome at the major site of transcription initiation is indicated by horizontal arrows.

data not shown), further suggesting that it may interact as a dimer.

Since there is a *Hha*I site (GCGC) in the −6 to −3 region of the promoter, we also examined whether methylation of the internal cytosine affected the affinity of the factors. Figure 5 (C and D) shows that modification of the probe with *Hha*I methylase decreased the yield of the cIBR (5C) and cIBF (5D) complexes with the TIS probe, although the affinity of cIBR was affected to a higher degree (14-fold versus 8-fold). Competition with TIS oligonucleotide and digestion of the probe with *Hha*I further indicated that the residual complex made with the methylated probe was indeed specific (Figure 5, C and D).

Figure 5E summarizes the footprint and the methylation interference data of cIBR and cIBF. It is striking that the region of interference is much shorter and asymmetrically located, with respect to the footprint. Since there is a significant degree of sequence redundancy in the DNaseI protected regions, it is possible that long footprints reflect the interaction of more than one complex of the factors (see Discussion).

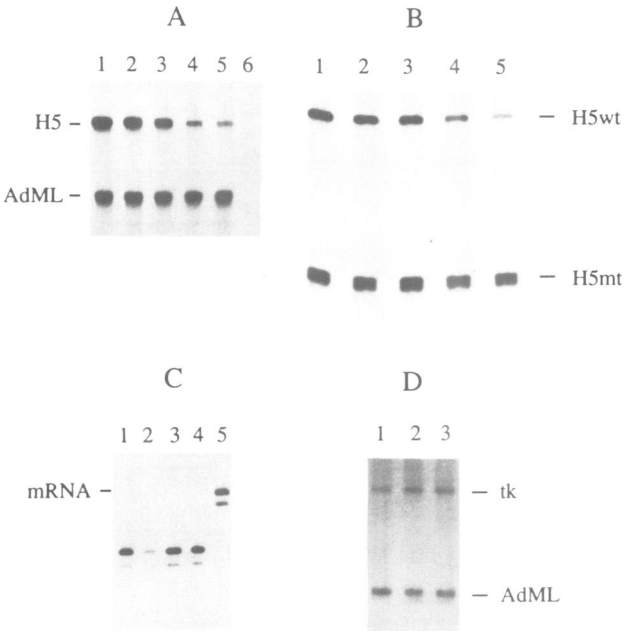

Fig. 6. *In vitro* repression by cIBR. (**A**) Transcription reactions contained 250 ng each of the cloned H5 gene (pSP65/2.5HB) cut with *Bst*EII and the major late promoter of adenovirus type 2 (pSmaF) cut with *Tth*111I. cIBR (second DNA affinity chromatography) was pre-incubated for 10 min with the templates before addition of the HeLa extract. Lanes 1 and 6, no cIBR; lanes 2–5, 0.6, 1.2, 2.4 and 4.8 molar excess of cIBR; lane 6, α-amanitin at 2 μg/ml was included in the assay. (**B**) Mutation of the cIBR binding site prevents repression by cIBR. 150 ng each of the wild type (wt) and mutant (mt) −95 to +72 H5 promoter (see Figure 7) were mixed and incubated with increasing amounts of cIBR as in (A). The lengths of the run-off transcripts are 350 (mt) and 640 (wt) nucleotides. Lane 1, no cIBR; lanes 2–5, increasing concentrations of cIBR (0.6–4.8 molar excess). (**C**) Primer extension of *in vitro* transcripts from circular templates containing wild type and mutant −95 to +72 *H5* promoters. Lanes 1 and 2, wild type promoter; lanes 3 and 4, mutant promoter. Lanes 1 and 3 no cIBR; lanes 2, 4, 5 molar excess of cIBR; lane 5, primer extension of total RNA from anemic erythrocytes (see footnote for further details). (**D**) cIBR has no effect when located upstream of the HSV1 *tk* promoter. 150 ng of plasmid DNA containing the *tk* promoter were mixed with equal amounts of AdML and preincubated with a 5 molar excess of cIBR before transcription. Lane 1, ptkCAT; lane 2, ptkCAT(4+); lane 3, ptkCAT(4−). ptkCAT(4+) and ptkCAT(4−) contain at −109 a tandem tetramer of TIS oligonucleotide in the direct and reverse orientation, respectively.

cIBR represses in vitro transcription from the H5 promoter

Since immature erythrocytes from anaemic animals proved to be unsuitable for gene transfer and cIBR has not been found in any of several cell lines examined (including HeLa), we approached the functional aspects of cIBR by *in vitro* transcription of the gene with HeLa nuclear extracts. Pre-incubation of linearized plasmid DNA containing the −95 to +72 *H5* promoter and the adenovirus major late promoter (AdML) with a 0.6, 1.2, 2.4 and 4.8 molar excess of purified cIBR resulted in a progressive inhibition of RNA polymerase II transcription specifically from the *H5* promoter (Figure 6A). The activity of the *H5* promoter dropped to 89, 63, 24 and 13%, taking that of the AdML as reference (Figure 6A, lanes 1–5) as the concentration of cIBR increased. Selective repression of *H5* transcription was independent on whether the two promoters were assayed individually (Figure 6A) or together (Figure 8) and a comparable degree of inhibition was observed when cIBR was mixed with the Hela extract before addition of the templates (Figure 8, and data not shown).

The fact that cIBR is a DNA binding protein made it likely that repression was mediated by the template-bound rather than the free protein, although the possibility of an indirect effect could not be ruled out. Therefore, a mutant *H5* promoter was constructed by introducing four nested transversions at positions −6 to −3 (Figure 7B). The mutation decreased the affinity of cIBR by a factor of five to 10 (although, interestingly, it did not affect that of cIBF, see Figure 7A). Figure 6B shows the results of *in vitro*

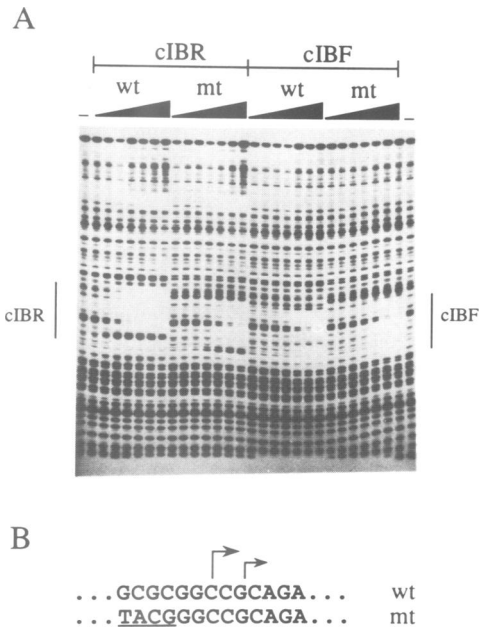

Fig. 7. Effects of a promoter mutation on cIBR and cIBF affinity. (**A**) DNase I footprinting reactions were carried out with wild type (wt) or mutant (mt) −95 to +72 *H5* promoter labelled at the 5′ end of the non-coding strand. Lane (−), no protein. The amount of cIBR (WGA fraction) used was 6.25, 12.5, 25, 50, 125, 250 and 500 ng. The amount of cIBF (first DNA affinity chromatography) was: 3, 6, 12, 25, 50, 100 and 200 ng. The relevant sequences of the wt and mt promoters are indicated in (**B**).

transcription of an equimolar mixture of the wild type (640 nucleotide transcript) and mutant template (350 nucleotide transcript) preincubated with increasing concentrations (0.6−4.8 molar excess) of cIBR. The activity of the wild type promoter was progressively reduced to 81, 67, 48 and 23% of the control with increasing amounts of cIBR, whereas that of the mutant promoter was not significantly affected (it decreased to 84% at the highest cIBR concentration). Primer extension results (Figure 6C) also showed that the *in vivo* major and minor transcription start sites were accurately used *in vitro* in the wild type and mutant promoters and that their rate of transcription was comparable in the absence of cIBR. No repression by cIBR was observed when the only GCGC site of the −95 to +72 promoter was methylated at −5 with *Hha*I methylase (data not shown, see Figure 5C). These results demonstrate that the DNA-bound form of cIBR is the effector of repression.

Negative regulation of transcription in eukaryotic systems is often mediated by factors bound upstream of the promoter (for reviews see Levine and Manley, 1989; Renkawitz, 1990). Therefore, it was of interest to determine the effect of cIBR when acting at a position different from that of the *H5* gene. This was examined by *in vitro* transcription of a HSV-1 *tk* promoter containing a tetramer tandem repeat of TIS oligonucleotide inserted at position −109. Figure 6D shows that at a five molar excess cIBR did not significantly affect the activity of the *tk* promoter, regardless of the presence and orientation of the inserted sequences, even though a control DNase I digestion confirmed that the cIBR boxes were occupied by the factor (data not shown). This suggests that the proximity of cIBR to the transcription start site may be a requisite for its effect.

cIBR inhibits assembly of the transcription initiation complex

Assembly of the transcription pre-initiation complex (fast starting complex) is thought to involve binding of TFIID to the TATA element (promoter commitment), followed by interaction of RNA polymerase II and TFIIB with the committed promoter (for review see Sawadogo and Sentenac, 1990). This pre-initiation complex is subsequently recognized by other general transcription and elongation factors that allow RNA polymerase II to start transcription in the presence of nucleoside triphosphates. Low concentrations of sarkosyl can be used to inhibit formation of the initiation complex without affecting the elongation rate of initiated polymerases (Hawley and Roeder, 1985). We made use of these observations to determine the step at which cIBR interferes with *H5* transcription.

To this end, the (−95 to +72) *H5* and control AdML templates were mixed and transcribed in a HeLa nuclear extract in the presence or absence of cIBR and Sarkosyl, as indicated in Figure 8. Addition of 0.025% sarkosyl had little effect on transcription from pre-assembled complexes (cf. lanes 1 and 5), whereas it completely inhibited transcription when added at the same time as the nuclear extract (Figure 8, lane 4). Note that the lower amount of transcripts in Figure 8 (lane 5) is probably due to inhibition by sarkosyl of additional initiation events, most clearly seen for the AdML promoter. When a five molar excess of cIBR was added together with the extract at the beginning of the incubation, transcription from the H5 promoter was inhibited

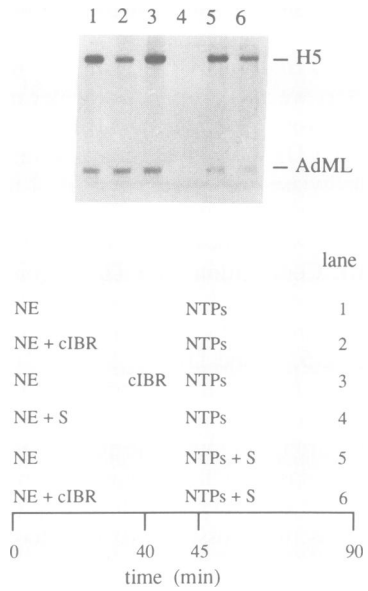

Fig. 8. cIBR interferes with assembly of the transcription initiation complex. A mixture of 150 ng each of −95 to +72 *H5* and AdML promoter was transcribed *in vitro* with a HeLa nuclear extract (NE) according to the experimental design indicated below the figure. Sarkosyl (S) was used at a final concentration of 0.025%, and cIBR at a molar excess of 5. NTPs, nucleoside triphosphates.

to 35% of the control, whether or not sarkosyl was added after a 45 min pre-incubation (Figure 8, lanes 2 and 6). On the other hand, cIBR had no effect on transcription from the AdML promoter. Since cIBR is released by the sarkosyl treatment, as judged by DNase I assays (not shown), these results indicate that cIBR interferes with assembly of the initiation complex of the *H5* promoter and not with a subsequent step of transcription. In accordance with this interpretation, cIBR had no effect when added after formation of the initiation complex (Figure 8, lane 3).

TFIID binding to the TATA element prevents cIBR binding

The footprints of nuclear proteins on the *H5* TATA element and the site of transcription initiation (Figure 1A) initially suggested that TFIID and cIBR could bind to the promoter in a mutually non-exclusive way. However, the possibility that protection of the TATA element was produced by a protein(s) unrelated to TFIID could not be ruled out. Due to the central role of TFIID on the assembly of the initiation complex, it was relevant to examine whether the purified factors showed any binding interference. Footprinting analysis using recombinant human TFIID (hTFIID) and purified cIBR indicates that binding of hTFIID to the TATA element (Figure 9, lane 3) induces a remarkable hypersensitivity of the flanking sequences from −47 to −31 and from −11 to +22, which include the cIBR binding site (lanes 4 and 5). Not surprisingly, binding of hTFIID prevented subsequent footprinting of cIBR (lanes 6 and 7) at concentrations that produce full protection when added alone (lanes 4 and 5). Interaction of cIBR with the TFIID−DNA complex induced, however, strong hypersensitivity at the boundary of the hTFIID and cIBR footprints. Conversely, binding of cIBR to the region of transcription initiation did not prevent subsequent footprinting

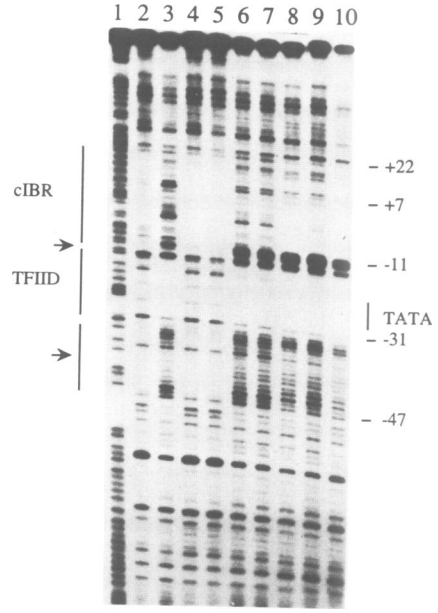

Fig. 9. hTFIID interferes with cIBR binding. DNase I footprints of the *H5* promoter labelled at the 5′ end of the coding strand. Lane 1, G+A ladder; lane 2, no protein; lane 3, 1.5 footprinting units (fu) of hTFIID; lanes 4–5, 7 and 14 ng of cIBR; lanes 6–7, 1.5 fu hTFIID (added in first) plus 7 and 14 ng of cIBR (added in second); lane 8, 7 ng cIBR (added in first) plus 1.5 fu of hTFIID (added in second); lanes 9–10, 1.5 fu of hTFIID plus 7 and 14 ng of cIBR added simultaneously. Hypersensitivity induced by factor binding is indicated by arrows.

of hTFIID (lane 8) and similar results were obtained when both factors were present simultaneously during binding (lanes 9 and 10). Interestingly, the cIBR footprint under the latter conditions is shorter (from −9 to +8) than in absence of hTFIID (Figure 9, cf. lanes 4 and 5, and 8–10). The shorter footprint covers the region of methylation interference (see Figure 5E), a finding that supports the view that the extended footprint of the farther downstream sequences represents a secondary site of cIBR interaction. Footprinting analysis of the non-coding strand produced a comparable set of data, indicating that the interactions described are not strand specific (data not shown). The evidence presented, therefore, strongly suggest that binding of TFIID prevents subsequent binding of cIBR, but not vice versa, in complete agreement with the *in vitro* transcription results presented in Figure 8.

Functional assays of cIBF

The effect of cIBF on *H5* gene transcription was examined by transfection of *H5*–CAT reporter genes into HD3 cells. Constructs containing the −95 to +72 *H5* promoter were methylated with *Hha*I methylase, an enzyme that will only modify the promoter at −5, decreasing the affinity of cIBF for the transcription start region by a factor of eight (see Figure 5D). To control for possible effects of methylation at *Hha*I sites elsewhere in the construct, we used the −95 to +72 promoter containing a mutation upstream of the transcription start sites that does not affect the affinity of cIBF but that removed the *Hha*I site of the promoter (Figure 7). Transfection of non-induced HD3 cells with methylated and non-methylated wild type constructs indicated

Table I. Effect of DNA methylation on *H5* promoter activity in non-induced (without enhancer) and induced (with enhancer) HD3 cells

H5 Reporter	*Hha*I methylase	Relative CAT activity	N[a]
Without enhancer			
p(5′)95	−	100[b]	6
p(5′)95	+	41	6
p(5′)95mt	−	100	3
p(5′)95mt	+	50	3
With enhancer			
p(5′)95AB*AS*	−	100[b]	2
p(5′)95AB*AS*	+	109	2
p(5′)95mtAB*AS*	−	107	2
p(5′)95mtAB*AS*	+	106	2

[a]Number of independent transfections.
[b]Averaged relative CAT activities are referred to that of the indicated constructs in each series.

that methylation reduced the activity of the promoter by a factor of 2.5 (Table I). However, the effect was not specific since transfection of the methylated mutant promoter also resulted in a similar decrease of CAT activity (Table I). We have also transfected *H5* reporter genes carrying the downstream enhancer (which has no *Hha*I methylation sites) in front of wild type and mutant −95 to +72 promoters and determined the effect of *Hha*I site methylation on their transcriptional activity in cells induced to differentiate. As was the case with the enhancerless constructs, no effect of methylation could be observed (Table I). The lack of even the non-specific decrease in activity of the methylated molecules is due to the presence of the enhancer rather than to cell differentiation, since the same behavior was observed in non-induced cells (data not shown).

Discussion

We have isolated two DNA binding proteins, cIBR and cIBF, that specifically recognize sequences spanning the major and minor transcription initiation sites of the histone *H5* gene. These proteins have different footprints (e.g. cIBR protects −10 to +19 and cIBF −8 to +23 of the coding strand), but make the same DNA contacts with a shorter region (−6 to +4), as inferred from methylation interference. This suggests that the DNA binding domains of cIBR and cIBF could be related and that the extended footprints reflect binding of the factors to adjacent sites, the −6 to +4 being their primary site of interaction. The shorter footprints obtained with a mutant in which the downstream sequences up to +5 were deleted supports this view (data not shown).

cIBR was purified from mature erythrocytes as a heterogeneous 75 kDa protein containing *N*-acetyl-glucosamine residues, a modification previously shown to occur in other transcription factors (Jackson and Tjian, 1988). The basis for the heterogeneity is not certain although it could be due to proteolysis, since the yield of the smaller component is variable and its proportion appears to increase with handling of the sample. Judging from DNase I footprinting and gel retardation assays, our data indicate that cIBR is present in mature and late polychromatic adult erythrocytes but not in several other cell lines examined, including erythroid (HD3), lymphoid (chicken MSB-1) and

fibroblasts (human HeLa, mouse L, and quail H-32), (unpublished observations). Therefore, the appearance of cIBR parallels the inactivation of the *H5* gene.

cIBF has been purified from transformed erythroid precursor cells (HD3) as a mixture of 68 and 70 kDa proteins with no affinity for WGA. Although both protein species were specifically crosslinked to oligonucleotides containing the cIBR/cIBF cognate sequence, further work is required to determine whether the 68 and 70 kDa proteins are related by degradation or modification, or if they are different gene products. cIBF-like DNA binding activities were also found in a number of non-erythroid cells, as inferred from DNA footprints and gel retardation assays with WGA binding and non-binding nuclear proteins (unpublished observations). Hence, cIBF appears to be an ubiquitous factor although cIBR and cIBF may not occur in the same cells.

Differences in tissue distribution, molecular weight, glycosylation, DNase I footprints and affinity for a mutant of the cognate sequence, appear to indicate that cIBR and cIBF are different gene products. However, the possibility that those differences are due to the glycosylation of cIBR cannot be dismissed. The lack of co-existence of cIBR and cIBF in the erythroid cells that were examined suggests that a transition between cIBF and cIBR occurs after the erythroblast stage. Hence, cIBR could be either a modified form of cIBF, or a late factor appearing as the cells approach maturity, following the down regulation of cIBF. Although additional work is needed to clarify the relationship between cIBR and cIBF, the fact that different DNA binding factors recognize related sequences has been previously documented for families of transcription factors, including OTF (reviewed by Schaffner, 1989), AP1/jun (Nakabeppu *et al.*, 1988), NFI/CTF (Santoro *et al.*, 1988; Rupp *et al.*, 1990), steroid/thyroid hormone receptors (Glass *et al.*, 1988; Umesono *et al.*, 1988), ATF/CREB (Hai *et al.*, 1989), and GATA (Yamamoto *et al.*, 1990).

Using a functional *in vitro* transcription assay we have shown that cIBR represses transcription of the histone *H5* gene. Repression was specific and required binding of the factor at the site of transcription initiation, since cIBR had no effect when located in an upstream position. Our studies indicate that cIBR somehow interferes with assembly of productive initiation complexes, but that it has no effect once the complexes have been formed.

Initiation of transcription from class II promoters requires several general transcription factors, in addition to RNA polymerase II and it has been demonstrated that binding of TFIID to promoters containing the TATA element is the first step in the formation of a transcription-committed complex (Sawadogo and Sentenac, 1990). Since TFIID is the only general transcription factor that displays specific DNA binding activity, we have examined whether cIBR interferes with promoter commitment. Our results suggest that while TFIID binding prevents the subsequent binding of cIBR, by what appears to be a conformational distortion of the cognate sequence, the opposite is not true. Therefore, cIBR does not interfere with promoter commitment but with a subsequent step in the formation of the initiation complex. Since binding of TFIIB to the TFIID–DNA complex results in a footprint on the region of transcription initiation (Buratowski *et al.*, 1989), we propose that cIBR prevents the interaction of TFIIB–RNA polymerase II with this region.

cIBR is the first documented example of a cellular eukaryotic factor that represses transcription by binding to the transcription start region. It can be easily imagined that if a balance between binding of TFIID and cIBR exists *in vivo*, this would permit a quick transition between full transcriptional activity and inactivity of the target genes. Regulation of cIBR activity *in vivo* could thus allow the rapid inactivation of genes otherwise endowed with all of the other necessary transcription factors. This mechanism of repression, widely used in prokaryotic systems (for review see Adhya, 1989), is used by certain viruses as autogenous regulation. This is the case of the SV40 T antigen (Rio and Tjian, 1983) and that of ICP4 (Muller, 1987), the product of an early HSV-1 gene, which all inhibit transcription of their own genes by binding to the transcription initiation sequences.

An increasing number of reports indicate that sequences around or immediately downstream of the transcription start site are important for gene activity, although the mechanism of action is poorly understood (e.g. Jones *et al.*, 1988; Ayer and Dynan, 1990; Garfinkel *et al.*, 1990; Nakatani *et al.*, 1990). A class of these sequences, the 'initiator' element, is found in some tissue specific genes devoid of discernible TATA elements and appears to be able to direct efficient and accurate basal transcription *in vitro* (Smale and Baltimore, 1989; Beaupain *et al.*, 1990; Pal *et al.*, 1991). A homologous initiator element is also found in promoters containing TATA boxes, namely the adenovirus IVa2 and major late and POMC. Although initiation complexes formed at either of the adenovirus elements are capable of supporting transcription, the presence of both motifs is required for efficient and accurate transcription (Carcamo *et al.*, 1991). cIBR is functionally unrelated to the initiator binding proteins since it was found in erythrocytes and not in cells that contain the initiator binding activity (HeLa). Furthermore, cIBR had no effect on transcription from the AdML promoter.

A different type of initiation elements and binding factors are involved in selection of the start site of transcription of the SV40 late promoter and the *DHFR* gene (Ayer and Dynan, 1988; Means and Farnham, 1990). cIBR/CIBF are also unrelated to these factors since, aside the lack of homology of the recognition sequences, cIBR did not bind to the relevant region of the SV40 late promoter (data not shown). Although we have not been able to show a transcriptional effect of *H5* promoters with lower affinity for cIBF, nevertheless it remains possible that this factor may cooperate in the selection of the start site of *H5* transcription.

Materials and methods

Cells

Chicken lymphoblastoma MSB-1 cells were grown as described (Renaud and Ruiz-Carrillo, 1986). *ts34*AEV-transformed CFU(E) chicken erythroid HD3 cells were grown at 35°C and 5% CO_2 in DMEM supplemented with 8% fetal calf serum and 2% chicken serum. Cells were induced to differentiate at 42°C in the presence of 15 μM H-7 (Seikagaku) (Zenke *et al.*, 1990) and the efficiency of differentiation was monitored by benzidine staining (Orkin *et al.*, 1975). HeLa cells were grown in suspension in DMEM and 10% fetal calf serum at 37°C and 5% CO_2.

HD3 cells (2×10^7) were transfected with 0.5–1.5 μg plasmid DNA in 1 ml of 25 mM Tris (pH 7.4), 135 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4 and 250 μg/ml DEAE–dextran, for 30 min at room temperature. Half of the cultures were induced to differentiate 16 h after transfection. Cell extracts for CAT assays were prepared 3 days after transfection.

DNA and oligonucleotides

pSmaF contains the major late promoter of adenovirus 2 (Weil *et al.*, 1979). pSP65/2.5HB carries the histone *H5* gene and flanking sequences (Ruiz-Carrillo *et al.*, 1983). p(5′)95 contains the −95 to +72 promoter of the *H5* gene fused to the CAT reporter gene and p(5′)95AB*AS* contains the downstream enhancer inserted upstream of the −95 promoter in the reverse orientation (Rousseau *et al.*, 1989). p(5′)95mt and p(5′)95mtAB*AS* correspond to the wild type constructs except that they carry a mutation of the cIBR binding site. They were constructed by replacing the sequences between −43 (*Sst*II) and −2 (*Not*I) by the product of a shot gun ligation containing the following pairs of complementary oligonucleotides: wt (−43) GGTCCGTG/GGCACGGACCGC, wt (−36) CCGCACCCTTA/TTTA-AGGGTGC, wt (−25) AATGCGTGCTG/ACCAGCACGCA, and mutant (−14) GTGGCACtacg/GGCCcgtaGTGCC. The longest product was ligated to the *Sst*II−*Not*I sites of the *H5* reporter genes.

p*tk*CAT contains the −109 to +51 HSV-1 thymidine kinase gene fused to the CAT reporter gene. ptkCAT(4+) and ptkCAT(4−) contain tandem tetramers of the cIBR wt oligonucleotide (−20 to +14) cloned at the upstream *Bam*HI site of ptkCAT in the direct and reversed orientation, respectively.

DNA was methylated with *Hha*I methylase (New England Biolabs) according to the manufacturer's instructions. *S*-adenosylmethionine was not included in mock methylation reactions. Oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer. The following oligonucleotides were used (only the sequence of the sense strand is given for the double stranded oligonucleotides): *H5* mRNA primer: CGGATG-TGGGGCTGCTCCTGC; ds cIBR wt (−20 to +14): AATTCGTGCTG-GTGGCACGCGCGGCCGCAGACGCAGCG; ds cIBR wt (−10 to +14): GCACGCGCGGCCGCAGACGCAGCG; ds cIBR mutant (−20 to +14): AATTCGTGCTGGTGGCACtacgttCCtCAGACGCAGCG; ds cIBR mutant (−10 to +14): GCACtacgttCCtCAGACGCAGCG; ds chicken *H5* NFI: GATCCACCGAGGGCTTGGCACAGCCCCAAG; ds SV40 AP1: GC-ATCTCAATTAGTCAGCAACC.

A cIBR DNA affinity column was prepared essentially as described (Chodosh *et al.*, 1986). The TIS oligonucleotide (−20 to +14) was oligomerized (average of 10mer) and the ends were filled with biotin 7-dATP (BRL) and dTTP using the Klenow fragment. The purified biotin labelled oligomers were bound to streptavidin−Sepharose (BRL). The concentration of bound DNA was 160 μg/ml of packed resin.

Nuclear extracts and factor purification

Nuclear extracts were prepared from isolated nuclei with 0.42 M KCl in buffer A (25 mM HEPES, pH 7.9, 20% glycerol, 3 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.2 mM EGTA, 6 mM 2-mercaptoethanol, 5 mM β-glycerophosphate, 0.5 mM PMSF, 50 μg/100 ml each of leupeptin and pepstatin) and concentrated by precipitation with 70% ammonium sulfate. The precipitates were solubilized in buffer A containing 0.1 M KCl, dialysed against the same buffer, cleared by centrifugation and stored in liquid nitrogen.

cIBR was prepared from a nuclear extract of 5×10^{11} mature hen erythrocytes. The extract was applied to a 4.5 ml wheat germ agglutinin−Sepharose 4B column (Pharmacia). The column was washed with 0.1 M KCl in buffer A containing 0.1% Nonidet P40. Bound proteins were eluted in the same buffer containing 0.3 M *N*-acetylglucosamine. A pool of 8 ml of the glycosylated proteins was mixed with 320 μg of sonicated salmon sperm DNA and applied to a 1.4 ml TIS affinity column. The column was eluted stepwise with buffer A containing 0.1, 0.25, 0.5 and 1.0 M KCl. The 0.5 M fraction containing cIBR was diluted with buffer A to 0.1 M KCl and further purified by a second round of DNA affinity chromatography.

cIBF was purified from a nuclear extract of 3×10^{10} HD3 cells. The extract was first applied to a 4.5 ml wheat germ agglutinin−Sepharose 4B column. The flow through was diluted to 0.2 M KCl in buffer A, applied to a heparin−agarose column (Pharmacia) and eluted with a 0.2−0.8 M KCl linear gradient in buffer A. The cIBF containing fractions (0.4−0.5 M KCl, estimated purification factor of 40) were pooled and dialysed against 0.1 M KCl in buffer A, mixed with 100 μg of sonicated salmon sperm DNA and applied to the cIBR affinity column. Elution was as described for cIBR. The 0.5 M KCl fraction containing cIBF was mixed with 80 μg of bovine serum albumin, dialysed against 0.2 M KCl in buffer A and reapplied to the affinity column for a second round of purification. cIBF activity eluted again in the 0.5 M KCl wash.

For preparative gel electrophoresis, 300 μl of DNA affinity purified cIBR was mixed with pre-stained molecular weight markers (Bio-Rad) and precipitated with 10 vol of 5% triethylamine, 5% acetic acid in acetone. After PAGE, proteins were eluted from gel slices for 16 h at 25°C in 250 μl of 50 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1% SDS, 5 mM dithiothreitol, 150 mM NaCl, 0.2 mM PMSF and 100 μg/ml of bovine serum albumin. Ten vol of acetone were added to the extract and the precipitated material was solubilized in 6 M guanidine hydrochloride and renatured as described (Hager and Burgess, 1980).

Proteins were analysed by SDS−PAGE (7.5%) and stained with silver (Morrissey, 1981). Protein concentration of purified fractions was estimated by comparison to stained molecular weight markers.

DNase I protection and gel retardation assays

Footprint reactions (20 μl) were carried out in 0.1 M KCl in buffer A containing 5−10 ng of ^{32}P-end labelled DNA (10^4−3×10^4 c.p.m.), 0−1 μg of poly(dAdT), 0−50 μg of nuclear protein or purified factors, 0−100 μg/ml bovine serum albumin, 0−400 molar excess of competing oligonucleotide, 50 ng pdN_5 (Pharmacia) and 0.1% Nonidet P40. After 30 min of incubation at 25°C, 1 μl of DNase I (10 ng to 1 μg) was added, and the reaction was stopped after 2 min by addition of SDS to 1%, EDTA to 25 mM, and proteinase K to 100 μg/ml. DNA was purified and analysed in 6% denaturing polyacrylamide gels.

DNA complexes with bacterial expressed human TFIID (Promega) were formed in the absence of competitor, essentially as described above, except that KCl was at 25 mM.

Gel retardation and methylation interference assays were carried out as described previously (Rousseau *et al.*, 1989). The samples were analysed in native 4% polyacrylamide gels.

UV crosslinking

The probe was prepared by hybridizing a 49mer oligonucleotide (−15 to +13 sequences of the *H5* promoter flanked by *Eco*RI and *Hin*dIII sites) to a 16mer complementary primer abutting the 3′ end. The primer was extended with Sequenase (USB, version 2.0) in the presence of 50 μM dATP, 50 μM dGTP, 50 μM 5-Bromo-dUTP, 5 μM [^{32}P]dCTP (NEN, 800 Ci/mmol). 4 ng of probe (0.8×10^6 d.p.m.) was incubated in a final volume of 50 μl with 2.5 μg of cIBF (heparin fraction) in buffer A containing bovine serum albumin (1 μg/μl) and 100 ng of poly(dAdT). Samples were irradiated at 302 nm for 30 min at 0°C, essentially as described by Chodosh *et al.* (1986). After crosslinking, the reaction mixtures were made 1.3 mM in $CaCl_2$ and incubated for 20 min at 37°C with 10 units of micrococcal nuclease and 11 μg of DNase I. Samples were analysed by SDS−PAGE (6.5%) and the gel was fixed and dried before autoradiography.

In vitro transcription

150−300 ng of linearized DNA template were incubated in a 20 μl reaction volume with 50 μg of HeLa nuclear extract in 12.5 mM HEPES (pH 7.9), 12% glycerol, 60 mM KCl, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 mM creatine phosphate, 0.5 mM each of ATP, CTP, UTP, 50 μM GTP and 10 μCi [α-^{32}P]GTP (specific activity 700 Ci/mmol, ICN). After 1 h at 30°C the nucleic acids were purified and analysed in 4% polyacrylamide denaturing gels. When circular substrates were used for transcription, the products of the reaction were analysed by primer extension using H5 mRNA primer, MuMLV reverse transcriptase (BRL) and RNasin (Promega) (Ruiz-Carrillo *et al.*, 1983) in 8% denaturing polyacrylamide gels.

Acknowledgements

We thank Dr G.Goodwin for a gift of HD3 cells and M.Lambert for synthesizing the oligonucleotides. A.G.-C. acknowledges an AIRE 91 exchange fellowship from C.I.R.I.T. (Generalitat de Catalunya), S.R. a studentship from the Fonds de la Recherche en Santé du Québec, and A.R.-C. a scholarship from the MRC (Medical Research Council of Canada). The research was supported in part by grants from the MRC and DGICYT (PM90−0032).

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Received on January 13, 1992; revised on February 10, 1992.