fected rGH promoters is strong in both the L cells (*8, 9*) and the hybrids. A similarly strong effect is exerted by GC1 on rGH promoter activity in vitro, although somewhat contradictory results have been obtained in transfection studies in which GC1 binding sites were deleted (*2, 4, 5*). It seems likely that both positive and negative mechanisms contribute to the extinction of expression of the rGH gene observed in hybrids. We have suggested (*8, 9*) that such a combination of positive and negative regulatory effects is required to generate the extremely strong (>10^8-fold) cell-type specificity of rGH expression (*13*).

Similar mechanisms may be involved in extinction of expression of other genes. This predicts, for example, that in lymphoid × fibroblast hybrids (*14*), the negative regulatory elements that flank the immunoglobulin heavy chain enhancer (*15*) should be activated. Similarly, expression of lymphoid-specific immunoglobulin gene promoter/enhancer binding factors (*16*) may be shut off in such hybrids.

Both positive and negative effects described here could arise from a single, simple mechanism. In particular, an L cell factor responsible for activating the negative effect of the rGH silencer could also act to shut off GC1 or GC3 expression, or both. Examples of such a factor may be provided by tentatively identified negative regulatory loci called tissue-specific extinguishers (*tse*'s). Expression of the appropriate *tse* is thought to be sufficient to shut off expression of tyrosine aminotransferase (*17*) or albumin (*18*) in liver × fibroblast hybrids. Similarly, a single *tse* could be sufficient for activation of the rGH silencer and repression of the rGH promoter binding proteins. Alternatively, if the two processes are mechanistically distinct, it might be possible to separate them genetically by approaches analogous to those used to identify the liver *tse*'s.

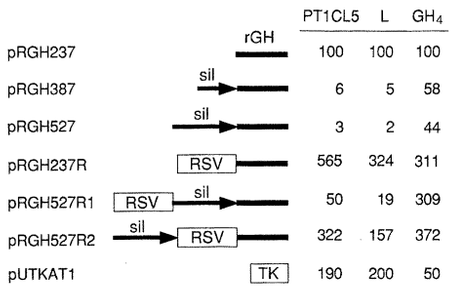

Fig. 3. Transient transfections of parental and hybrid cell lines. Relative CAT expression is presented as percent of the level of expression directed by pRGH237 (*8*). Results with pUTKAT1, which contains the herpes virus TK promoter fused to CAT (*25*), are included for comparison of promoter activity. The solid bar represents the 237-bp rGH promoter–containing fragment, the arrows represent additional 5′ flanking sequences (−237 to −387 in pRGH387, and −237 to −527 in other plasmids) that contain the silencer. The open boxes represent fragments containing the RSV enhancer or TK promoter, as indicated. LTK^- (L) cells were transfected by use of DEAE dextran, and both PT1CL5 and GH_4 cells were transfected using calcium phosphate as described (*8*). Absolute levels of CAT enzymatic activity obtained with pRGH237 averaged 6.1, 9.7, and 2% of total chloramphenicol converted per 100 μg of protein per 4 hours, for LTK^-, pituitary cells, and hybrids, respectively. The effects of variations in transfection efficiency were eliminated by normalizing CAT expression levels to levels of hGH expression directed by the cotransfected control plasmid pXGH5 (*26*). Results represent averages of at least nine independent transfections, in which relative expression varied by less than 20%. Results similar to those obtained with PT1CL5 were also obtained with the independent hybrid line PT1CL6.

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27. We thank J. W. Harney for help with transfections. Supported by a grant from Hoechst AG to Massachusetts General Hospital and a fellowship from the Fonds de la Recherche en Santé du Québec (S.G.).

21 March 1988; accepted 1 July 1988

Binding of a Cytosolic Protein to the Iron-Responsive Element of Human Ferritin Messenger RNA

TRACEY A. ROUAULT, MATTHIAS W. HENTZE, S. WRIGHT CAUGHMAN, JOE B. HARFORD, RICHARD D. KLAUSNER

The human ferritin H chain messenger RNA contains a specific iron-responsive element (IRE) in its 5′ untranslated region, which mediates regulation by iron of ferritin translation. An RNA gel retardation assay was used to demonstrate the affinity of a specific cytosolic binding protein for the IRE. A single-base deletion in the IRE eliminated both the interaction of the cytoplasmic protein with the IRE and translational regulation. Thus, the regulatory potential of the IRE correlates with its capacity to specifically interact with proteins. Titration curves of binding activity after treatment of cells with an iron chelator suggest that the factor acts as a repressor of ferritin translation.

FERRITIN IS AN INTRACELLULAR PROtein that sequesters iron. When cells are treated with iron, ferritin mRNA shifts from messenger ribonucleoproteins (mRNPs) to polysomes and becomes actively translated (*1, 2*). This regulation occurs in the absence of changes in ferritin mRNA levels (*3*). A 35-nucleotide region in the 5′ untranslated region (5′ UTR) of human ferritin H chain mRNA contains an element that is both necessary and sufficient for translational regulation by iron (*4, 5*). Placement of this IRE in the 5′ UTR of mRNAs encoding chloramphenicol acetyltransferase

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.

(CAT) or human growth hormone results in the transfer of translational regulation by iron to these hybrid mRNAs. Deletion of the IRE from the ferritin H chain mRNA eliminates all translational regulation (*4*). The human ferritin H chain IRE and similar sequences found in the 5′ UTR of other ferritins have the capacity to assume a stem-loop structure (*5*, *6*). Two single-base deletions in the IRE have been shown to completely eliminate translational regulation (*7*). The IRE may provide a site for interaction with a trans-acting factor, which either represses or activates translation in response to changes in intracellular iron levels. In this report we demonstrate specific binding of one or more cytosolic proteins to the human ferritin H chain IRE. Experiments with an IRE containing a single-base deletion show that the ability of the IRE to confer iron regulation of translation correlates with its ability to bind the cytosolic factor.

The human ferritin H chain IRE can form a characteristic stem-loop structure (Fig. 1A, left). The stem contains one or more unpaired bases including an unpaired cytidine six nucleotides 5′ of the loop. The sequence of the loop of all known ferritin IREs is CAGUGX (*7*, *8*). We have generated synthetic IREs in which the bases of the stem are altered such that a base-paired stem structure is maintained, though the bases of the stem differ significantly from those of native ferritin IREs. These synthetic elements confer iron-dependent translational control to nonferritin genes (*8*). Thus, the exact nucleotide sequence of the IRE stem does not appear to be critical.

In order to study the specificity of the interactions of the IRE and cytosolic factors, several DNA fragments were subcloned into vectors from which RNA could be transcribed in vitro. A synthetic oligonucleotide was used to direct the synthesis of an IRE (Fig. 1A, right) within the 5′ UTR of chimeric transcripts (*7*). The lower part of the stem was provided by Bam HI restriction enzyme recognition sequences. A single-point deletion in this IRE that removes the conserved 5′ C of the loop (C_{-165}) was also synthesized.

The function of the synthetic IREs shown in Fig. 1A was assessed by CAT enzyme activity with constructs described previously (*7*). The CAT construct containing no IRE (designated −IRE) demonstrated no response to iron manipulation, but when the 26-nucleotide synthetic IRE (Fig. 1A, right) was inserted just 5′ to the CAT coding region, CAT activity increased in response to the iron source (hemin) and decreased in response to iron chelation (desferrioxamine) (Fig. 1B). We have previously shown that mRNA levels for constructs containing the IRE do not change in response to these iron manipulations (*4*). In contrast, when a synthetic IRE lacking cytidine residue −165 was inserted into the 5′ UTR of chimeric transcripts (designated +IRE ΔC_{-165}), no iron-dependent regulation was observed. Thus, cytidine −165 is a critical residue for IRE function in translational control.

The ability of cytosolic factors from K562 cells, a human erythroleukemia cell line, to interact with the IRE was assessed by means of a gel retardation assay similar to the one first described by Leibold and Munro (*9*). Two RNA probes containing functional IREs were used (*10*). One probe (designated 5′ UTR) contains a portion of the human ferritin H chain transcript and includes the native ferritin IRE in a transcript of 128 bases. The other probe (designated IRE) is an RNA fragment of 111 bases containing the synthetic IRE shown in Fig. 1A, right. After incubation of either of these RNA probes with cytosolic S100 extracts (*11*) from human K562 cells, a complex was formed that resulted in the retarded migration of the labeled RNA in the gel (Fig. 2). Both probes were retarded to the same position in the gel after ribonuclease (RNase) T1 digestion and addition of heparin. In contrast, a probe derived from the first 150 bases of the β-globin mRNA failed to form such a complex. The specificity of complex formation was demonstrated by the failure of the cytosolic extract to retard the migration of an RNA probe containing an IRE-like sequence lacking the functionally critical cytidine residue (−165) of the loop (designated ΔIRE).

The specificity of the retardation complex was further supported by competition studies. The formation of the specific complex with the 5′ UTR probe was completely abolished by a 500-fold molar excess of unlabeled 5′ UTR probe. It was also eliminated by an excess of unlabeled IRE probe.

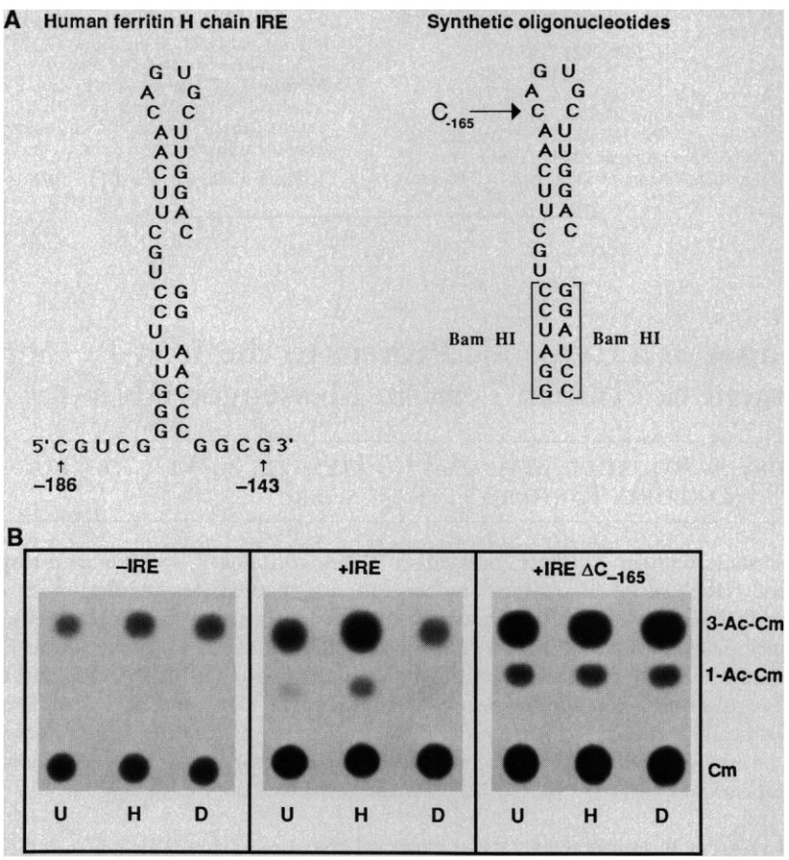

Fig. 1. A single-base deletion destroys ability of IRE to produce translational regulation. (**A**) The sequence of human ferritin H chain IRE and a synthetic oligonucleotide are depicted showing predicted secondary structure (*16*). The arrow in the synthetic oligonucleotide indicates the position of C_{-165}. (**B**) Translational regulation of CAT expression requires an intact IRE. Enzymatic activity of CAT expression (*17*) was assessed with a plasmid (*5*) lacking the synthetic IRE (designated −IRE), a plasmid containing the intact IRE (designated +IRE) and a plasmid in which C_{-165} was deleted (designated +IRE ΔC_{-165}). The positions of the acetylated CAT products (3-Ac-Cm and 1-Ac-Cm) and chloramphenicol (Cm) are indicated. Equal amounts of lysates from untreated cells (U) or cells treated with the iron source hemin (H) or the iron chelator desferrioxamine (D) were assayed.

In contrast, the β-globin probe did not compete with the 5′ UTR probe for complex formation, and the ΔIRE produced only a minor decrease in the intensity of the complex. The retardation of the IRE probe was also effectively blocked by excess unlabeled IRE probe or 5′ UTR probe but again was unaffected by excess unlabeled β-globin or ΔIRE probes. These findings indicate that the interaction is both specific and saturable. A similar complex was observed when cytosolic extracts of human liver were used (*12*). The formation of the complex in vitro does not require capped transcripts, since the probes used here were not capped. The factor responsible for retardation of the IRE-containing RNA requires intact protein since the gel retardation band was eliminated by treatment of the complex with proteinase K (Fig. 2). We will refer to this factor as a protein, though formation of the retardation complex may also require other factors in addition to proteins.

A simple model for the function of the regulatory protein would suggest that iron-dependent translational regulation correlates with binding of the protein to the IRE. If the protein functions as a repressor of translation, then cells which have been depleted of intracellular iron would be expected to contain more repressor activity. Translation of ferritin mRNA is markedly decreased in vivo by treatment of cells with an iron chelator. We have observed that the intensity of the signal of the specific complex in the gel retardation assay is proportional to the amount of the cellular protein added. The gel retardation assay was employed to assess the effect of iron manipulation on the activity of the IRE-binding protein. After overnight treatment of K562 cells with desferrioxamine (100 μ*M*), gel retardation assays were done with increasing amounts of probe (Fig. 3). Independent cell lysates from desferrioxamine-treated cells reproducibly displayed binding activity four- to fivefold as high as that of lysates from untreated cells at each probe concentration (Fig. 3B). Treatment of cells in duplicate with a lower concentration of desferrioxamine (25 μ*M*) produced an intermediate increase in binding activity. The correlation between increased IRE binding activity in vitro and decreased ferritin biosynthesis in vivo suggests that the protein functions as a repressor of ferritin translation in iron-depleted cells. The effect of treatment of cells with desferrioxamine cannot be reproduced by exogenous addition of desferrioxamine to cell lysates (*13*).

The IRE appears to function in translational control as the recognition site for a cytosolic protein. Little is known about the binding of cytosolic factors with specific RNA regulatory sequences. Recently, a gel retardation assay was used to demonstrate the binding of rat liver cytosolic factors to the 5′ UTR of rat ferritin L chain transcript (*9*). Two specific gel retardation complexes were observed in that study, in contrast to the single complex seen here.

We have demonstrated that a correlation exists between the function of an IRE in translational control and its ability to interact with a cytosolic protein. Two findings

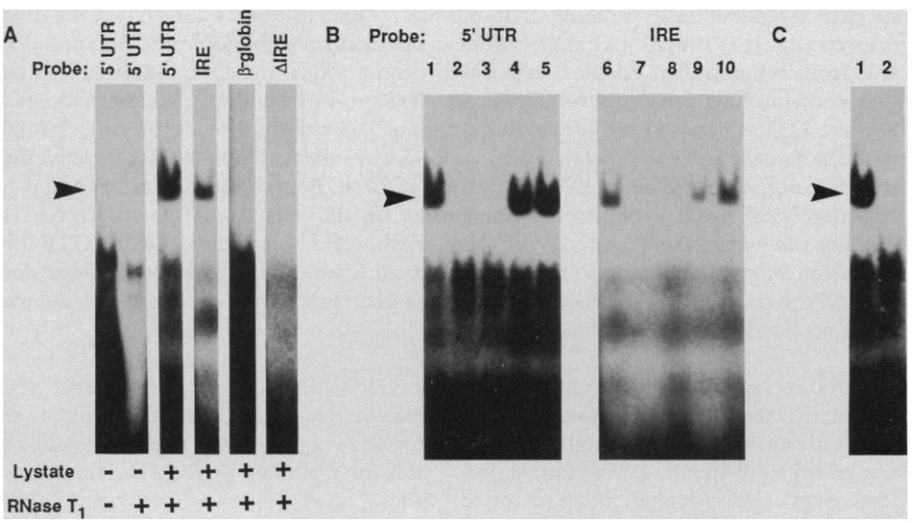

Fig. 2. The IRE from the 5′ UTR of ferritin interacts specifically with cytosolic protein. (**A**) The indicated probe (10^5 cpm, 3×10^8 cpm per microgram) was incubated for 30 min at 25°C with 40 μg of S100 cytosolic extract prepared (*11*) from human K562 cells. RNase T_1 (1 U, Calbiochem) was then added and the incubation allowed to continue for 10 min. Heparin sulfate (Fisher Scientific, Fairlawn, New Jersey) was then added to a concentration of 5 mg/ml and the incubation continued for an additional 10 min. Samples were then applied to a 4% acrylamide nondenaturing gel (*9*) and electrophoresis was performed for 3 hours at 8 V/cm. An autoradiograph of the resultant gel is shown with the position of the IRE-specific retardation complex indicated by the arrow. (**B**) Experimental protocol was as in (A) with ^{32}P-labeled 5′ UTR or IRE probes. The indicated unlabeled RNA (150 to 250 ng) was added to the incubation mixture immediately prior to addition of the ^{32}P probe. The competitor was, for lanes 1 and 6, none; for lanes 2 and 7, 5′ UTR; for lanes 3 and 8, IRE; for lanes 4 and 9, ΔIRE; and for lanes 5 and 10, β-globin. (**C**) Experimental protocol was as in (A) with ^{32}P-labeled 5′ UTR probe. Before electrophoresis, proteinase K (1 μg/μl, Boehringer Mannheim) was added to the reaction mixture used in lane 2, and the incubation was allowed to proceed for an additional 10 min. ΔIRE contains the synthetic IRE in which C_{-165} is deleted.

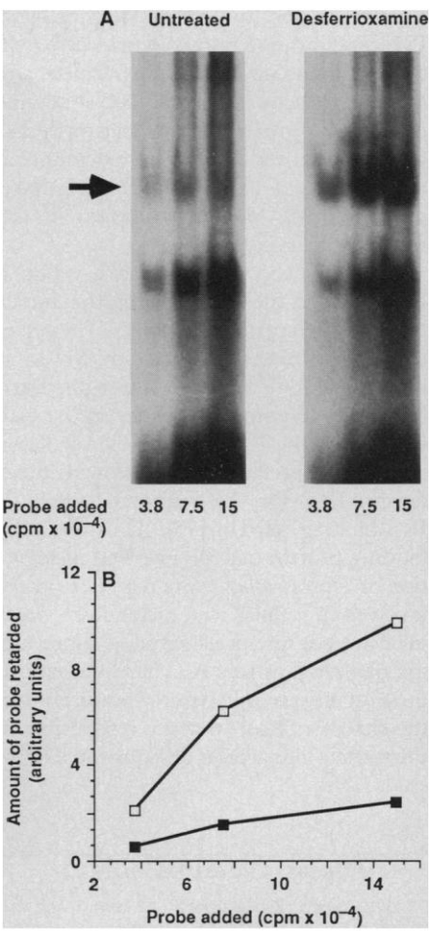

Fig. 3. Treatment of K562 cells with an iron chelator increases the IRE binding activity of cytosolic extracts. (**A**) K562 cells were untreated or treated with 100 μ*M* desferrioxamine for 16 hours at 37°C. S100 cytosolic extracts were prepared and the gel retardation assay was performed as in Fig. 2 with ^{32}P-labeled 5′ UTR probe. Increasing amounts of probe (3.8×10^4 to 15×10^4 cpm) were added to 13 μg of cytosolic extract and increasing amounts of the specific retardation complex detected (arrow). (**B**) The amount of probe retarded in each lane was determined with an LKB Ultrascan XL Enhanced Laser Densitometer with computer-assisted peak integration. Values are expressed in arbitrary units of peak area. □, Desferrioxamine; ■, untreated.

allow us to make this correlation. First, an altered sequence (ΔIRE) is unable to confer iron-dependent translational control. Second, this altered sequence is incapable of interacting with cytosolic protein as evidenced by its failure either to form a retardation complex or to significantly compete with an IRE in the formation of that complex. This strict correlation of binding and function is analogous to the interaction of the R17 coat protein with an mRNA stem-loop structure in which a single base change in the loop eliminates binding (*14*).

The role of the IRE-binding protein in the function of the IRE was further established by experiments in which intracellular iron levels were manipulated. When K562 cells were treated with the iron-chelator desferrioxamine, the specific activity of the IRE-binding protein was increased four- to fivefold, a finding that is in agreement with the biosynthetic rates of IRE-containing mRNAs in untreated- and chelator-treated cells. Leibold and Munro have demonstrated the loss of an ultraviolet cross-linked protein-ferritin mRNA complex in cells treated with iron (*9*).

We have recently identified a group of IREs within the 3′ UTR of the human transferrin receptor mRNA (*8*). The region containing these IREs was shown to be responsible for iron-dependent regulation of transferrin receptor mRNA levels. We have found that the RNA of this 3′ regulatory region can specifically compete with the 5′ ferritin IRE for the interaction with the IRE-binding protein (*15*). Thus, the IRE-binding protein may be involved in regulation of expression of more than one protein involved in cellular iron metabolism. Many unanswered questions remain concerning the observations reported here. Answers to these questions will require purification of the factor or factors that are responsible for the specific interactions demonstrated in this study.

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10. The 5′ UTR probe was cloned from a Hae III–Eag I fragment representing bases −209 to −124 of ferritin H chain mRNA (*5*) into Hind III–Xma III sites of the pGEM 3-blue (Promega) polylinker after partial fill-in of the gap with the Klenow fragment of DNA polymerase I. The resultant plasmid was linearized with Eco RI and RNA transcripts produced by SP6 polymerase. The IRE probe was cloned from the previously described construct L5 (+26-nucleotide oligomer)-CAT (*7*). A 5′ blunt-ended Bgl I–Xba I fragment was gel-isolated and cloned into the Hind III–Xba I sites after Klenow fill-in of the Hind III site of the plasmid vector pGEM-3 zf(+) (Promega). The same approach was used to generate the ΔIRE probe from the plasmid designated Δ−165 (*7*). Both constructs contain ferritin promoter sequences and the first six bases of the ferritin 5′ UTR in addition to the IREs. Sequences were confirmed by using a modification of the dideoxy chain termination method of sequencing. Riboprobes were generated with SP6 RNA polymerase. β-globin transcripts were generated from a β-globin cDNA cloned into pSP64 [A. R. Krainer, T. Maniatis, B. Ruskin, M. R. Green, *Cell* **36**, 993 (1984)] and were linearized at an Acc I site 150 bases into the cDNA. Riboprobes were generated by the use of SP6 RNA polymerase.
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17. The plasmid designated −IRE corresponds to the L5-CAT plasmid described in (*5*). Transient transfections of plasmids and subsequent determinations of CAT activity were performed as described (*5*).

18. We gratefully acknowledge contributions of J. Casey, D. Koeller, J. Barriocanal, and A. Dancis to this work. S.W.C. was supported by the Dermatology Branch, National Cancer Institute, NIH. We thank E. Perry for her excellent work in preparation of this manuscript.

27 April 1988; accepted 27 June 1988

Transcription Factor OTF-1 Is Functionally Identical to the DNA Replication Factor NF-III

EDWARD A. O'NEILL, COLIN FLETCHER, CHRISTOPHER R. BURROW, NATHANIEL HEINTZ, ROBERT G. ROEDER, THOMAS J. KELLY

Octamer transcription factor–1 (OTF-1) and nuclear factor III (NF-III) are sequence-specific DNA binding proteins that activate transcription and DNA replication, respectively. It is shown here that OTF-1 is physically and biologically indistinguishable from NF-III. This conclusion is based on the following observations. First, the two proteins have identical mobilities by SDS–polyacrylamide gel electrophoresis. Second, OTF-1 binds to the adenovirus origin of DNA replication at the same site and with the same affinity as NF-III. Third, OTF-1 can substitute for NF-III in activating the initiation of adenovirus DNA replication in vitro. Fourth, the ability of OTF-1 to stimulate viral DNA replication is dependent on the presence of an intact NF-III binding site within the origin of replication. Fifth, NF-III can substitute for OTF-1 in activating in vitro transcription from the human histone H2b promoter. These data suggest the possibility that NF-III/OTF-1 is a protein that functions in both cellular DNA replication and transcription.

IT IS BECOMING INCREASINGLY APPARENT that the regulation of all nucleic acid synthesis may occur through the function of sequence-specific DNA binding proteins. Transcription promoters are repressed or activated (*1, 2*) and origins of DNA replication become functional (*3*) in the presence of proteins that recognize and bind specific DNA sequences. The identification and characterization of such proteins are the first steps in developing an understanding of how these proteins perform their regulatory role.

Fractionation of the adenovirus DNA replication reaction in vitro (*4*) has allowed the identification of several proteins that can participate in the replication of DNA within virus-infected HeLa cells. Three of these proteins are viral in origin: the 80-kD preterminal protein (pTP), the 140-kD DNA polymerase (Ad Pol), and the 72-kD DNA binding protein (DBP) (*4*). The remaining proteins are cellular in origin: a topoisomerase, NF-II (*5*), and two sequence-specific DNA binding proteins, NF-I (*6, 7*) and NF-III (*8, 9*). We have recently purified NF-III

E. A. O'Neill and T. J. Kelly, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
C. F. Fletcher and R. G. Roeder, Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021.
C. R. Burrow, Department of Molecular Biology and Genetics and Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
N. Heintz, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021.