The XLR Gene Product Defines a Novel Set of Proteins Stabilized in the Nucleus by Zinc Ions

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Abstract. The major product of the XLR (X-chromosomal, lymphocyte-regulated) locus is found to be a 30-kD nuclear protein with a relatively short ($t_{1/2} \cong 2$ h) half-life. Together with its stage- and tissue-specific pattern of expression, this suggests a role for this protein in the regulation of differentiation in T and B lymphocytes. Interestingly, the XLR protein almost completely leaches out of the nucleus after lysis of cells in low salt buffer, but is stabilized in that location by metal cations, particularly Zn^{++}. This stabilization is reversible by chelating agents (*o*-phenanthroline, EDTA) which also release a number of other polypeptides in addition to XLR. These results suggest that XLR represents a novel class of nuclear proteins, and that cations such as zinc may play a role in the localization of these proteins in the nucleus.

As part of an effort to identify novel genes and their products which are important in lymphocyte differentiation and/or function, we first identified transcripts of the XLR locus using the methodology of subtractive hybridization (Cohen et al., 1985*a*). XLR consists of a family of sequences located on the X chromosome, and appears to be expressed largely in lymphoid cells (Cohen et al., 1985*a,b*). This expression is developmentally regulated both in the T- and the B-cell lineage where only the most mature cells transcribe this locus (Cohen et al., 1985*a*; and Siegel et al., 1987). Although there appear to be many copies of this gene, only one potentially functional transcript has thus far been found (Siegel et al., 1987). A full-length cDNA clone, pM1, contains a single open reading frame of 208 amino acids (Siegel et al., 1987) with a predicted molecular mass of 25 kD. Analysis of the amino acid sequence revealed a highly charged molecule with no significant stretches of hydrophobic character, indicating that XLR is neither a membrane-associated nor a secreted protein. In addition, weak (14%) but significant identities have been found between XLR and intermediate filament proteins, particularly lamins A and C. The carboxy-terminal half of the molecule also has an α helical character with an uncharged face, further suggesting that XLR is a distantly related member of the intermediate filament family of sequences (Siegel et al., 1987). Clearly an important step in determining the function of the XLR is to determine its location within cells that express it. Towards this end, we have produced quantities of XLR protein in the procaryotic expression system of Rosenberg and his colleagues (Devare et al., 1984; Ferguson et al., 1984;

Watt et al., 1985), and prepared rabbit antisera against it. Analyses using these antisera show that XLR is nuclear in location, and has a short half-life. Cell fractionation studies also reveal a novel dependence on metal ions, especially zinc, for this nuclear localization. Although this effect has not been reported for any known protein, a variety of other (unknown) nuclear proteins appear to have these same characteristics. Thus, XLR appears to typify a class of nuclear proteins which are stabilized in the nucleus by metal ions, and may illustrate some new principle of nuclear architecture or be significant in a regulatory context.

Materials and Methods

In Vitro Expression and Purification of the pM1 Gene Product

Construction of the pOTS-M1 Plasmid. The 5′ end of the pM1 cDNA was digested with exonuclease III to a position just 3′ of the initiation codon, and the ends were made blunt with mung-bean nuclease. The cDNA was inserted between the filled-in Nde I site and the Hind III site of the pUC8 vector in order to recreate a Nde I site. The correct sequence was checked according to the procedure of Maxam and Gilbert (Maxam and Gilbert, 1980). The Nde I-Sal I fragment containing the complete coding region of the pM1 cDNA was ligated together with the Bgl II-Nde I fragment and the Xho I-Bgl II fragment of the pOTS vector (Devare et al., 1984).

Induction of XLR Expression in Bacterial Cells. AR120 cells (a cI⁺Ind⁺ derivative of N99) transformed with pOTS, or with the recombinant plasmid pOTS-M1, were grown in Luria-Bertani broth at 37°C to an OD_{600} of 0.7 when nalidixic acid (40 μg/ml) was added. At the indicated time-points, aliquots of these cells were taken, were washed, and were resuspended in Laemmli reducing SDS-PAGE sample buffer (Laemmli, 1970). The equivalent of 25 μl of the original culture was then loaded onto a 15% SDS-PAGE. Staining was with Coomassie blue.

Purification of the XLR Protein. A bulk culture of pOTS-M1-transformed cells was induced with nalidixic acid as described above. After 4 h, cells were pelleted and resuspended in TENG buffer (50 mM Tris, pH 8,
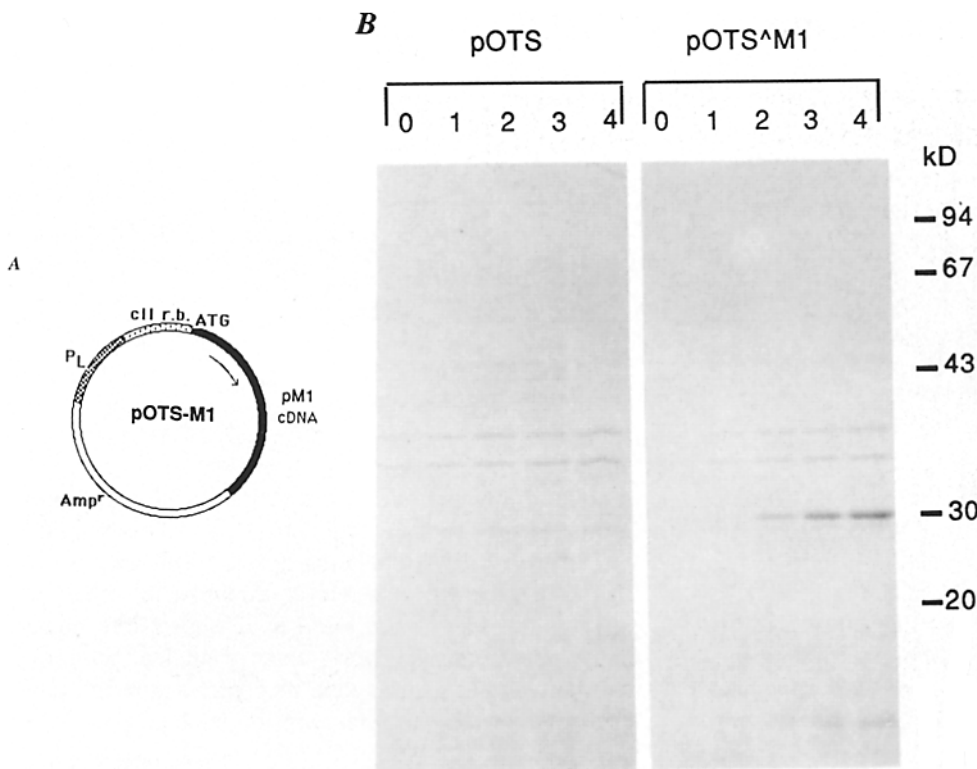
Figure 1. In vitro expression and purification of the pM1 gene product. (*A*) Map of the pOTS-M1 plasmid. O_1 and P_1 λ phage left operator and promoter; *cII r.b.*, cII ribosome binding site. (*B*) Induction of XLR gene expression: AR120 cells transformed with the pOTS-M1 plasmid or with pOTS were grown at 37°C and induced in the presence of nalidixic acid. Aliquots were harvested at 1-h intervals from 0 to 4 h (lanes *0–4*) and the induced proteins were analyzed by SDS-PAGE. (*C*) 2D-gel electrophoretic comparison of purified XLR material (*top*) and of in vitro–translated pM1 gene product (*bottom*).

0.5 mM EDTA, 0.3 M NaCl, 5% glycerol). The cell walls were permeabilized in the presence of lysozyme (1 mg/ml) followed by addition of NP-40 0.2%. After DNAse I treatment, cells were washed in TENG buffer, and resuspended in Laemmli reducing sample buffer. This material was loaded onto a 15% preparative SDS-PAGE. The gel was stained with 0.25 M KCl. The band corresponding to the pM1 protein was cut out and electroeluted. The resulting material was extensively dialysed against 1 mM octyl-glucoside in PBS, then against PBS only, and a sample (1 × 10^{-3} OD_{280} unit) was analyzed by two-dimensional (2D)[1] gel electrophoresis (O'Farrell, 1975) and silver staining (Merril et al., 1981). For comparison, the pM1 cDNA was transcribed from the SP6 RNA polymerase promoter in the pGemini vector (Melton et al., 1984), then was translated in vitro in the presence of [^{35}S]methionine (Krieg and Melton, 1984) using rabbit reticulocyte lysate, and analyzed by 2D gel electrophoresis.

Preparation of Antibodies

New Zealand white rabbits were immunized twice subcutaneously with 50 μg of purified XLR protein. Antibodies to XLR were assayed by ELISA using alkaline phosphatase–conjugated goat antibodies to rabbit IgG. Antibodies were then affinity purified by passage through a column of Sepharose coupled to XLR protein.

Immunofluorescence Assay

Cultured cells were coated onto poly-L-lysine–treated glass coverslips and were fixed for 15 min with 2% formaldehyde in PBS-3% sucrose. The cells were permeabilized with 0.2% Triton X-100 in PBS and incubated with 10 μg/ml of affinity-purified anti-XLR antibody (for the XLR staining). Control antibody was always included and consisted of the purified preimmune IgG at the same concentration. It showed no staining (not presented here). After 1 h at room temperature, the excess antibody was washed off and the cells were incubated with rhodamine-conjugated goat F(ab)'2 antibodies to rabbit Ig coupled to rhodamine lissamine. For the anti-Ig staining, goat anti-Ig (F(ab)'2) antibodies (anti-IgM for BAL 17 and anti-IgA for MOPC 315) coupled to fluorescein were used simultaneously. Hoechst 33258 staining of nuclei was by standard incubation and wash conditions (Hilwig and Gropp, 1972).

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1. *Abbreviation used in this paper:* 2D, two dimensional.

Metabolic Labeling and Fractionation of Cultured Cells

Cells (2 × 10^6 in 1 ml) were incubated for 6 h at 37°C in methionine-free RPMI 1640 culture medium (or phosphate-free DME) supplemented with 5% fetal calf serum, 2 mM glutamine, 2 × 10^{-5} M 2-mercaptoethanol, and 100 μCi/ml of [^{35}S]methionine (or 0.5 mCi/ml of [^{32}P]orthophosphate). The cells were washed in cold PBS and were lysed in 10 mM Hepes buffer, pH 7.6, plus 10 mM NaCl, 3 mM $MgCl_2$, and 0.4% NP-40 with or without Zinc(II) or other divalent cations. The nuclei were spun down at 1,600 g for 8 min. Supernatants constituted cytoplasmic fractions. Nuclei were resuspended in 10 mM Hepes, pH 7.6, 0.5 M NaCl, 10 mM EDTA, and were sonicated. Insoluble material was then removed from this nuclear fraction by centrifugation at 16,000 g for 15 min. In some experiments, after lysis of the plasma membrane, the nuclei were centrifuged one or several times through a cushion of 30% sucrose. For nuclease treatment and extraction with chelators, the nuclei were resuspended in nuclease buffer (10 mM Hepes, pH 7.6, 25 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$). The appropriate concentration of micrococcal nuclease was determined in pilot experiments. After 10 min at 4°C, nuclei were pelleted. Supernatants were harvested (extracted fractions). Nuclei were resuspended in 10 mM Hepes, pH 7.6, 0.5 M NaCl, 10 mM EDTA, and treated as above.

Immunoprecipitation Assay

Samples were incubated with 1 μg of anti-XLR affinity-purified antibody or of preimmune IgG for 1 h at 4°C. The resulting immune complexes were recovered on protein A–Sepharose beads, were washed in 0.1 M sodium borate buffer pH 8.1, 1 M NaCl, 0.1% Tween 20 followed by 40 mM Hepes, 0.1% SDS, eluted by boiling in reducing buffer, and subjected to electrophoresis through a 15% SDS-PAGE.

Results

Expression of an XLR Gene Product and Generation of Antisera

As part of our effort to understand the function of the XLR locus, we sought to develop serological reagents for the pro-
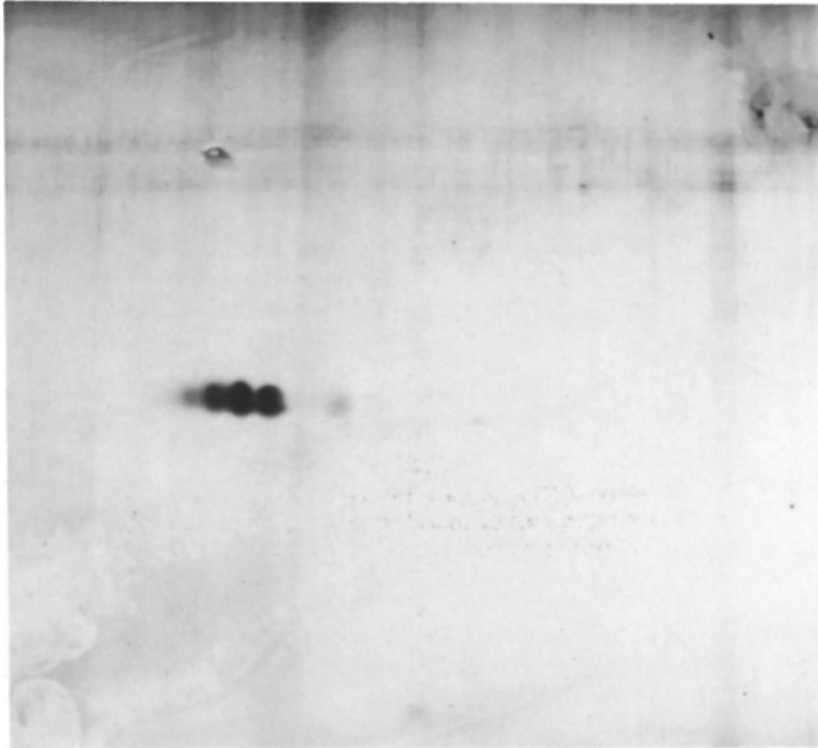
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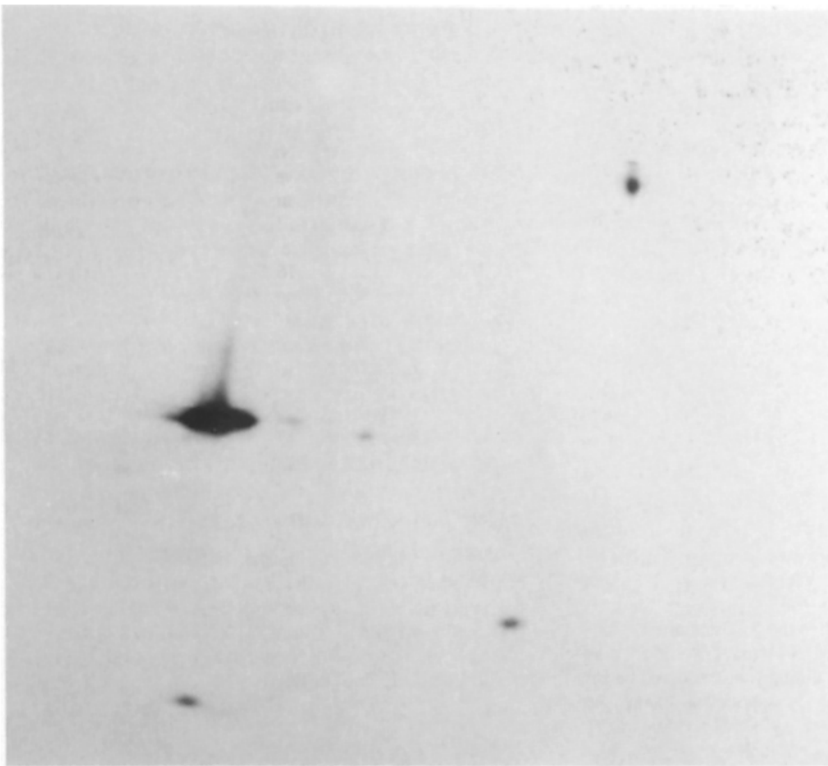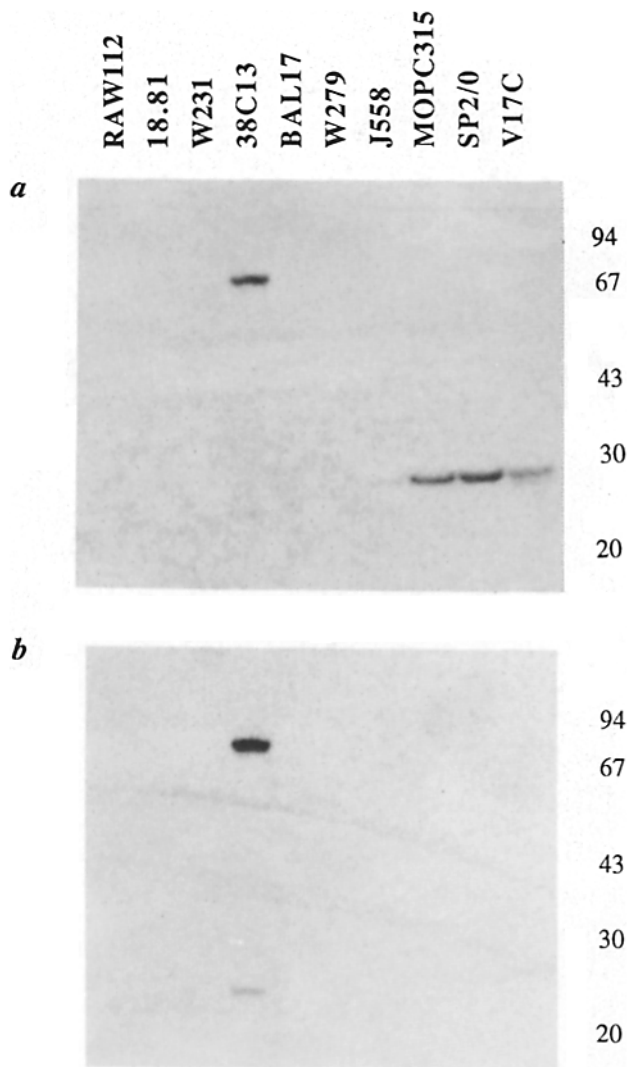acidic   IEF   basic

a

SDS-PAGE

b

SDS-PAGE

Figure 2. Identification of the XLR gene product in mouse lymphoid cell lines. B-cell tumor lines at various stages of differentiation (see text) were metabolically labeled with [³⁵S]methionine, washed, and lysed with NP-40. Nuclei were pelleted and supernatants were incubated with affinity-purified anti–XLR antibodies (*a*) or preimmune Ig (*b*). Immune complexes were adsorbed on protein A–Sepharose beads, washed, and subjected to electrophoresis on 15% SDS-PAGE.

tein and to localize it within cells that express it. To this end, we first expressed the pM1 cDNA by using the pOTS vector. This plasmid and its former version, pAS1, have successfully supported the production of large amounts of several unfused eucaryotic proteins, including c-*myc* (Watt et al., 1985), adenovirus E1A (Ferguson et al., 1984), and v-*sis* (Devare et al., 1984). It features the left promoter of the λ phage with N utilization sites for an efficient and controlled transcription and the Shine-Dalgarno sequence of the cII gene for active translation. The pM1 cDNA was deleted down to the AUG codon and fused in frame to the Nde I site present in the Shine-Dalgarno sequence (Fig. 1 *A*). This construction was transferred into AR 120 bacterial cells. After induction by nalidixic acid, aliquots of cells were harvested at successive time-points and were analyzed by denaturing SDS-PAGE. As

shown in Fig. 1 *B*, a prominent molecular species of an apparent molecular mass of 30 kD was specifically expressed in bacteria containing the recombinant plasmid pOTS-M1, but not in the cells transformed with the pOTS vector alone. Its molecular mass is identical to that of the product translated from SP6 RNA polymerase transcripts with rabbit reticulocytes in vitro (see below). It is somewhat higher than the predicted molecular mass (25 kD) as deduced from the nucleotide sequence. The discrepancy between the observed and the predicted molecular masses has been reported for other proteins (Hope and Struhl, 1985) and may be due to the variable ability of different proteins to bind to SDS. This material was prepared on a larger scale by detergent extraction of induced bacteria, preparative SDS-PAGE, excision, and electroelution of the XLR band. The purity of this sample was tested by 2D gel electrophoresis (O'Farrell, 1975) and silver staining (Fig. 1 *C, top*). Comparison with the XLR pM1 product gene translated in vitro (Fig. 1 *C, bottom*) shows an identical migration pattern with a slightly acidic isoelectric point (6.5) and a similar microheterogeneity, indicating that this material is homogeneous within the sensitivity limits of our silver staining technique.

Identification and Regulated Expression of an XLR Gene Product in Mouse B Cell Lines

Protein purified in this manner was used to immunize rabbits. Sera were collected and antibodies were purified over an affinity column of XLR protein coupled to Sepharose beads. These antibodies were used to detect XLR gene product(s) in mouse cells. A panel of B-cell tumor lines representative of different stages of maturation (pre-B, B, and plasma cells) were labeled metabolically with [³⁵S]methionine and cell lysates were immunoprecipitated with anti–XLR antibodies. Fig. 2 shows that all of the four plasma cell (Ig secreting) lines included in the study (MOPC 315, SP2/0, J558, and V17C) expressed an XLR-immunoreactive peptide with the same apparent molecular mass as the bacterial pOTS-M1 product and the in vitro-translated pM1 protein. Also, the MOPC 315 XLR gene product migrates on a 2D gel in an identical manner to these two peptides (data not shown). This indicates that plasma cell lines express a gene closely related to pM1 gene, if not the pM1 gene itself.

In contrast to myeloma cells, the four B-cell lymphoma lines tested (WEHI 231, 38C13, BAL 17, WEHI 279), two pre-B-cell Abelson virus-transformed lines (RAW112, 18.81), and the pre-B-cell lymphoma 70Z/3 (data not shown) do not express the XLR protein. These immunoprecipitation data also correlate with an immunofluorescence assay (see below). Altogether, these results demonstrate that a protein closely related to that predicted by the pM1 open reading frame is expressed in a stage-specific manner by terminally differentiated immunoglobulin-secreting B-cell tumor lines as a group and not by cells representing earlier stages of differentiation. This correlates well with RNA expression data obtained previously (Cohen et al., 1985*b*).

Nuclear Localization of XLR

To localize the XLR protein at the subcellular level, cells were fixed and stained with anti–XLR antibodies followed by rhodamine-conjugated goat antibodies against rabbit immunoglobulins. Fig. 3 compares two cell lines: BAL 17, which
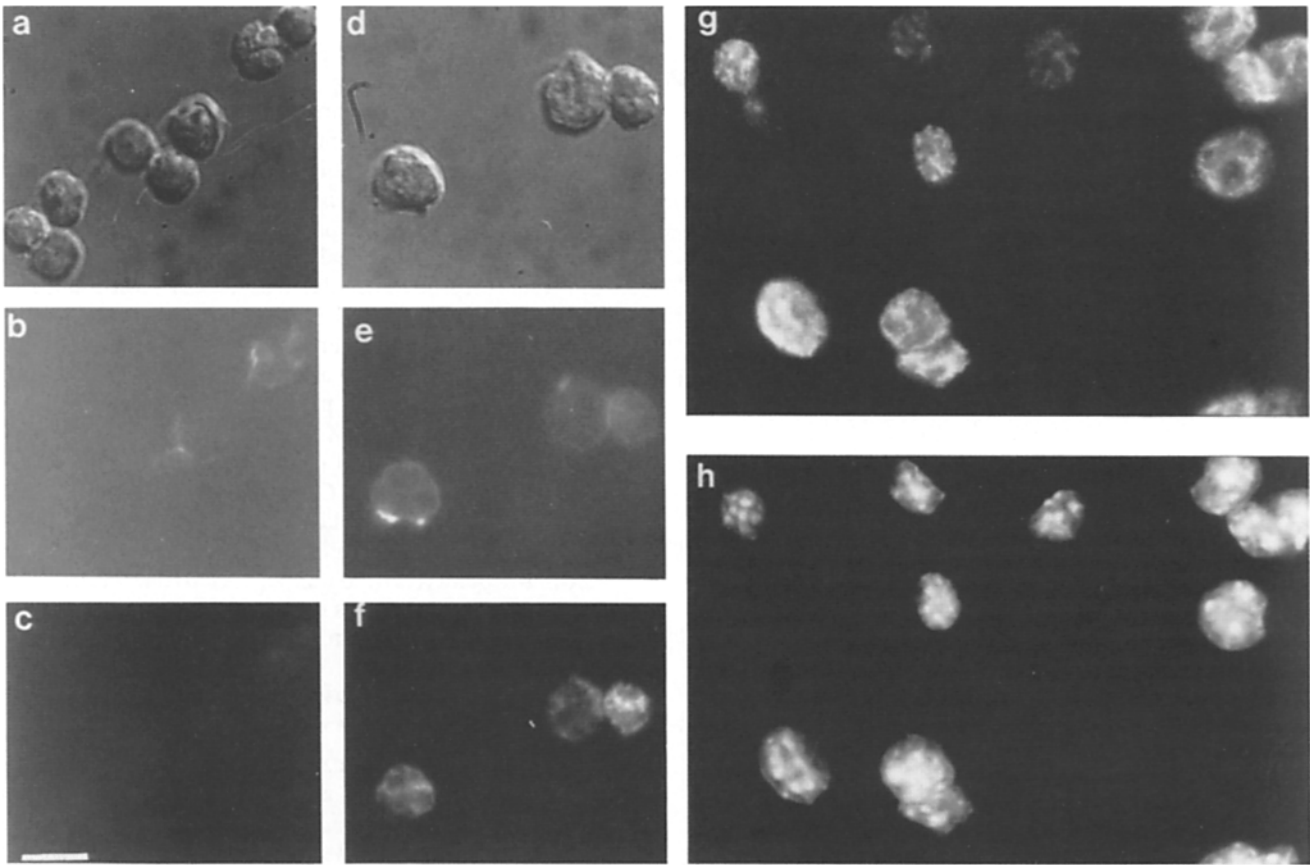
Figure 3. Nuclear localization of the XLR gene product. BAL 17 (*a*, *b*, and *c*) and MOPC 315 (*d*, *e*, and *f*) cells were compared with respect to anti-Ig (cytoplasmic and surface) staining (*b* and *e*) and anti-XLR staining (nuclear) of the same cells (*c* and *f*). The anti-Ig staining was visualized with fluorescence and the anti-XLR stain with rhodamine (see Materials and Methods). *g* shows anti-XLR staining of MOPC 315 cells with rhodamine compared with Hoechst staining of these same cells in *h*. Bar, 20 μm.

stains negatively, and MOPC 315, which is positive for XLR. Fig. 3, *a* and *d* are phase-contrast micrographs of BAL 17 and MOPC 315 cells, respectively, with the panels immediately below them (*b* and *e*) showing staining with antiimmunoglobulin antibody, which principally stains the cytoplasm. Fig. 3, *c* and *f*, below that, show the same cells stained with anti-XLR antisera, which stains only the nuclei of the MOPC 315 cells. To further emphasize that this is nuclear staining, Fig. 3 *g* contrasts anti-XLR staining of MOPC 315 cells with that of a DNA-specific fluorescent dye (Hoechst 33258). These data show clearly that XLR is in the nucleus of MOPC 315 cells, as has been found for all positive cell lines analyzed thus far (data not shown). The whole nucleus stained in a heterogeneous manner (Fig. 3, *f* and *g*). This is in contrast to that of antilamin antibodies which give a rim-like staining pattern (McKeon et al., 1986), and is reminiscent of nuclear proteins such as c-*myc* or c-*fos*.

Stabilization by Zinc Ions

In further contrast to lamins and nuclear scaffolding proteins (Nelson et al., 1986), the XLR peptide was consistently recovered in the cytoplasmic fraction after lysis of the plasma membrane using standard conditions, including neutral pH, low salt, magnesium chloride, and a nonionic detergent (NP-

40). Modification of several parameters that were shown to affect the localization of some nuclear proteins did not alter XLR behavior. Thus, the use of a mild detergent, octyl-glucopyranoside, or lysis by hypotonic shock and passage of the cells through a syringe needle, was not critical as has been reported for the SV-40 T antigen (Jones and Su, 1985), c-*myc* (Eisenman et al., 1985; Evan and Hancock, 1985), and c-*myb* (Boyle and Baluda, 1987; Boyle et al., 1985). The concentration of NaCl or its replacement by KCl did not modify XLR segregation in the cytoplasmic fraction either. The temperature at which the lysis is performed is another parameter that influences the nuclear location of c-*myc* (Eisenman et al., 1985; Evan and Hancock, 1985) and heat shock proteins (Littlewood et al., 1987), but the incubation of cells at 37°C before lysis did not have any effect on XLR localization. However, the addition of certain divalent metals did have an effect as shown in Fig. 4 *a*. Thus, cobalt and copper supported a significant retention of XLR inside the nucleus, but zinc was most efficient of all, with the majority of the protein remaining in the nuclear fraction. In contrast, calcium, magnesium, or manganese ions had no effect. This favors the idea of a specific role for zinc different from that of calcium or of copper which have also been reported to affect the stability of nuclear structure (Lebkowski and Laemmli, 1982*a,b*; Lewis and Laemmli, 1982). As shown

a

b
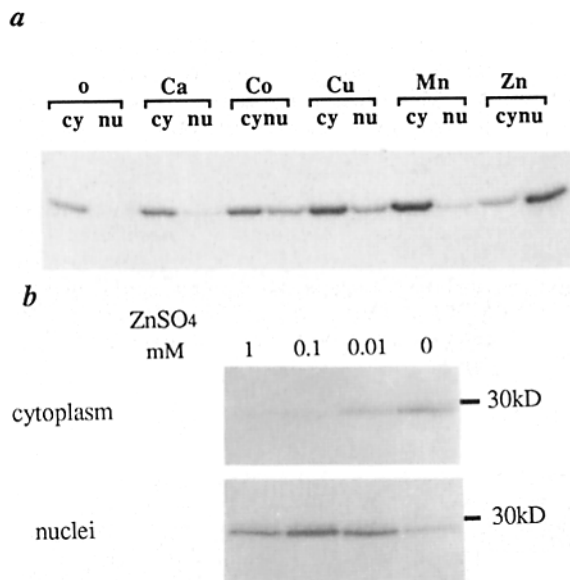
Figure 4. The role of Zinc (II) in the nuclear location of XLR. MOPC 315 cells were metabolically labeled as before and lysed in the presence of (a) 1 mM of specific divalent cations (Ca, CaCl₂; Co, CoCl₂; Cu, CuSO₄; Mn, MnSO₄; Zn, ZnSO₄; or (b) various concentrations of ZnSO₄ as indicated. Cytoplasmic and nuclear fractions from each sample were immunoprecipitated with anti–XLR antibodies and analyzed by SDS-PAGE.

Figure 5. Determination of the half-life of the XLR protein. MOPC 315 cells were labeled with [³⁵S]methionine for 2 h, then chased with cold methionine-containing medium, then lysed in the presence of ZnSO₄. Cytoplasmic and nuclear fractions were precipitated with anti–XLR antisera and analyzed by SDS-PAGE. The autoradiograph of the gel was analyzed with an LKB ultroscan XL densitometer and the area corresponding to the peak of each band was integrated (in absorbance units per mm²). Area measurements for the nuclear peaks are: 2.348 (t = 0 h), 0.749 (t = 2 h), 0.392 (t = 4 h), and for cytoplasmic peaks: 0.674 (t = 0 h), 0.409 (t = 2 h), and 0.083 (t = 4 h).
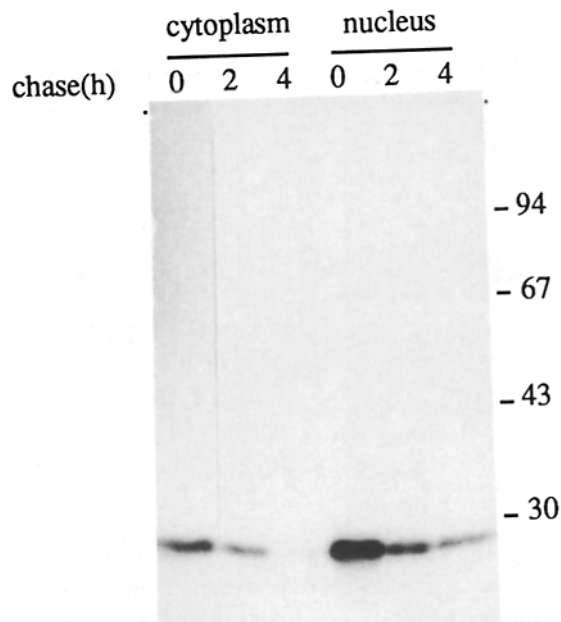
in Fig. 4 *b*, the concentration of zinc required for this effect ranges between 0.1 and 1 mM and does not change when lysis is performed in the presence of octylglucoside or by simple hypotonic lysis (not shown). This last result rules out a possible chelating effect of NP-40. The molarity at which we see this effect is comparable to the intracellular concentration of zinc (Milne et al., 1985; Whitehouse et al., 1982), although substantially higher than that of free zinc ions which may be in the nanomolar range (Metcalf et al., 1985).

XLR Has a Relatively Short Half-life

The half-life of XLR was determined by a pulse-chase experiment (Fig. 5). The nuclear fraction undergoes an exponential decay corresponding to a half-life slightly shorter than 2 h. The data about the cytoplasmic fraction may be more difficult to interpret as the respective contributions of the peptide traveling toward the nucleus after its synthesis and the leakage out of the nucleus occurring at the breakage of the cells has not been investigated.

### Reversibility with Chelating Agents

To analyze further the role of zinc, in a preliminary experiment we sought to extract the XLR peptide by washing the nuclei repeatedly in a zinc-free buffer, with the idea of later using the nuclei as an ion-exchange column from which XLR could be eluted along a decreasing gradient of zinc. The outflow of XLR was monitored by immunoprecipitation of the supernatant of each extraction step. As expected, when the control lysis was performed in the absence of zinc, most, if not all, of the protein was found in the cytoplasmic fraction and none was left in the nuclear pellet (Fig. 6 *a*). When zinc was included in the primary lysis buffer, only a small amount

of XLR leaked out of the nuclei (Fig. 6 *b*). Surprisingly though, five subsequent washes in zinc-free buffer, a procedure that decreases the concentration of zinc by many orders of magnitude, failed to extract the XLR protein from the nuclear fraction. An equivalent experiment consisted of spinning the cells, after lysis in the presence of zinc, through a sucrose cushion in a zinc-free buffer (Fig. 6 *c*). Here again, the XLR protein remained firmly associated with the nuclei. This suggests that the initial lytic event profoundly disturbs the nuclear structure, at least as far as the XLR protein is concerned. If zinc is present during this critical step, then a steady state seems to be reached rapidly. This is reminiscent of the behavior of the c-*myb* protein whose nuclear location depends on the type of detergent and of the ionic strength of the lysis buffer (Boyle and Balluda, 1987), although metal ions do not affect the nuclear location of another nuclear oncogene product, c-*myc* (Evan and Hancock, 1985).

To determine whether the nuclear retention was irreversible, perhaps the result of aggregation or precipitation of XLR within the nucleus, we next tried extraction procedures using chelating agents or nucleases. In these experiments, MOPC 315 nuclei were prepared in the presence of zinc, spun through a sucrose cushion, and were resuspended in a neutral buffer (10 mM Hepes pH 7.6, 10 mM NaCl, 3 mM MgCl₂) with various additives. As shown in Fig. 7 *a*, 0.5 M NaCl or micrococcal nuclease (or DNAse I and RNAse A, data not shown), or both in combination, failed to release XLR or released only a small quantity of the XLR protein

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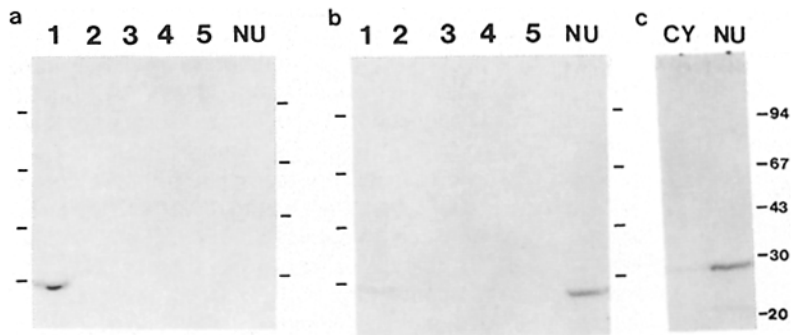
Figure 6. The presence of Zn(II) is required during the primary lytic event only. Metabolically labeled MOPC 315 cells were lysed (*a*) in the absence or (*b*) and (*c*) in the presence of 1 mM ZnSO₄. Nuclei were pelleted and resuspended in zinc-free 10 mM Hepes buffer, pH 7.6, 10 mM NaCl, 3 mM MgCl₂ buffer. (*a* and *b*) This procedure was repeated five times or (*c*) nuclei were spun directly through a cushion of 30% sucrose in zinc-free buffer. Supernatants were harvested as well as the last nuclear pellet, were treated as in Fig. 4, and were immunoprecipitated with anti–XLR antibodies.

out of the nucleus. Among chelators, EDTA, which binds strongly to most divalent cations, was able to extract a minor amount of XLR out of the nuclei. Another class of chelators, *o*-phenanthroline and its derivatives, is particularly interesting because it binds zinc and copper with high affinity but has a low affinity for magnesium and calcium. However it, by itself, failed to elute the XLR protein. Most interesting though was the synergistic effect of these chelators and salt, leading to a significant release of the XLR protein out of the nuclei. This synergy is particularly striking in the case of *o*-phenanthroline and salt since these two reagents separately failed to extract any XLR protein at all.

Other Nuclear Proteins Stabilized by Zinc

To evaluate how general this effect of zinc on the location of nuclear proteins could be, nuclei were prepared by lysis in the presence of zinc and were extensively washed in order to decrease the background of salt-extractible proteins. After

the last wash, nuclei were resuspended either in 0.5 M NaCl buffer or in the same buffer plus 5 mM *o*-phenanthroline. The composition of the extracted proteins was analyzed by SDS-PAGE and silver staining. As shown in Fig. 7 *b*, a large fraction of the nuclear proteins was released at first exposure to 0.5 M NaCl. Three additional washes with salt alone eluted little material (the equivalent of 20 times more nuclei was loaded on Fig. 7, lanes 2–5 compared with lane 1). However, addition of *o*-phenanthroline resulted in the release of additional proteins. Their molecular mass varied from 30 up to 200 kD. Also their number appeared to be discrete (within the limits of detection of our silver staining technique, i.e., 100–200 pg of XLR protein per millimeter of band width). This level of sensitivity did not allow us to visualize the XLR protein, indicating therefore that *o*-phenanthroline-extractible proteins are present in variable quantities in the nucleus. Our extraction protocol was carried out with addition of 0.5 M NaCl. At this fairly high concentration of salt, most DNA-binding proteins are dissociated from
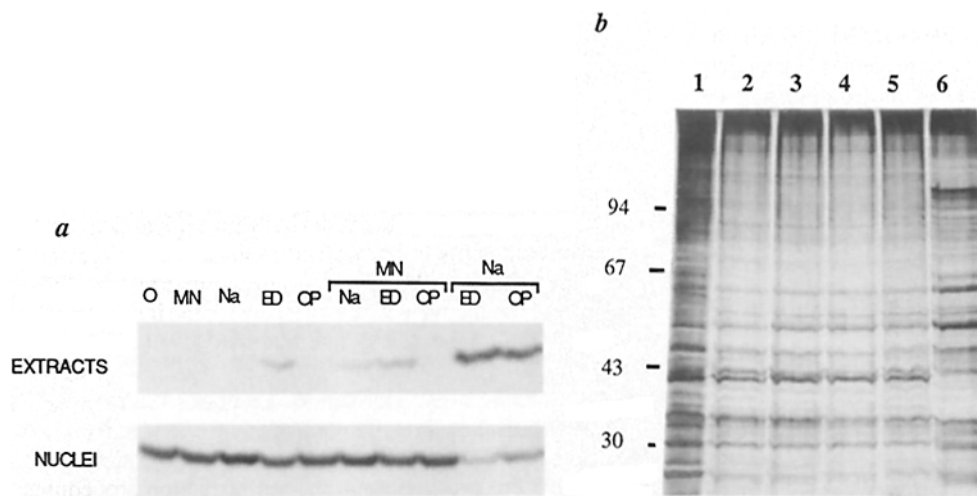
Figure 7. *o*-Phenanthroline extraction of XLR and of other nuclear proteins. (*a*) Nuclear extraction of XLR. In vivo labeled MOPC 315 cells were lysed in nuclease buffer (see Materials and Methods) containing 1 mM ZnSO₄ and 0.4% NP-40. Nuclei were spun through a sucrose cushion, were resuspended in 10 mM Hepes buffer, pH 7.6, 25 mM NaCl, 3 mM MgCl₂, and were incubated with or without various reagents (*Na*, 0.5 M NaCl; *MN*, 0.15 U/µl micrococcal nuclease; *ED*, 5 mM EDTA; *OP*, 5 mM *o*-phenanthroline). The appropriate concentration of micrococcal nuclease was determined in pilot experiments. After 10 min at 4°C, nuclei were pelleted and supernatants were harvested (extracted fraction). Nuclei were resuspended in 10 mM Hepes, pH 7.6, 0.5 M NaCl, 10 mM EDTA, and were sonicated. The extracted and the nuclear fractions were immunoprecipitated with anti–XLR antibodies and analyzed by SDS-PAGE as previously described. (*b*) *o*-Phenanthroline extraction of nuclear proteins. MOPC 315 nuclei were prepared in the presence of Zn(II) as described above. They were then resuspended in 10 mM Hepes, pH 7.6, 0.5 M NaCl, 3 mM MgCl₂, and were spun again through a 30% sucrose cushion in 10 mM Hepes, pH 7.6, 0.5 M NaCl, 3 mM MgCl₂. This procedure was repeated four times. Each time, the upper layer was collected (extracted fractions, lanes *1–4*). After the last centrifugation, nuclei were resuspended in 10 mM Hepes, pH 7.6, 0.5 M NaCl, 3 mM MgCl₂, in the absence (fraction *5*) or the presence (fraction *6*) of 5 mM *o*-phenanthroline and were pelleted at 16,000 *g* for 15 min. For each extraction step, the equivalent of 5 × 10⁴ (lane *1*), 1 × 10⁶ (lanes *2–5*), and 2.5 × 10⁵ (lane *6*) nuclei was analyzed by 12% SDS-PAGE and silver staining.

their substrate. The significance of this obligatory synergy between salt and chelators is not known although the salt dependence is most likely due to accessibility. The small amount of protein release seen in each lane is probably due to a small fraction of the nuclei lysing at each step. It should be mentioned that addition of zinc during the first lytic step considerably increased the resistance of nuclei which otherwise were rapidly disrupted by this treatment.

Discussion

B lymphocyte maturation is one of the best known systems of cell differentiation and yet very little is known about what types of genes initiate and maintain the phenotypically distinct stages. In terms of actual numbers, it has been estimated that only 200 genes distinguish B cells from T cells and that only 50–100 genes differ between tumor lines representing the B cell (surface immunoglobulin) and plasma cell (secreted immunoglobulin stages) (Davis et al., 1982; Hedrick et al., 1984; Turner, A., and M. Davis, unpublished observation). As many of these later species presumably represent structural proteins, it can be argued that XLR will be one of a small cohort of nuclear proteins specifically activated in this last stage of B cell differentiation. As mitogen-activated B cells (but not resting lymphocytes) are positive for the XLR protein in the spleen (Garchon, H.-J., unpublished observation), the stage specificity that we see in cell lines (Fig. 2) does not appear to be an artifact of transformation. Thus, the XLR gene product(s), because of its strict specificity and nuclear location, may play an important regulatory role in B cell differentiation. Assigning a function to XLR by analogy is difficult, however, as it does not fit into any known category of nuclear protein. Thus, while the homology to lamins is significant but distant, the fluorescent-staining pattern is not at all similar. It does not appear to be a DNA- or RNA-binding protein as the sequence has no known DNA-binding motif (homeo boxes, zinc fingers, etc.) and the protein is not released by nucleases. We conclude that there is no particular precedent for this type of protein in the nucleus, and that it represents a novel class of molecules.

In this respect, it is all the more intriguing that physiological concentrations of zinc govern whether or not XLR remains in the nucleus, and that a number of other proteins have these same characteristics. Two possible explanations might explain this effect.

(a) XLR and other proteins could be zinc-binding proteins. Withdrawal of zinc upon chelation might therefore result in conformational changes which destabilizes some association in the nucleus causing these proteins to leach out. This putative zinc binding could not be due directly or indirectly to a zinc-finger motif (Berg, 1986; Miller et al., 1985) both because XLR has no such sequence and because "zinc fingers" bind zinc ions very tightly (K_d 10^{-14} M), and even trace amounts of zinc in buffers have an effect. However, many proteins without zinc fingers bind this ion, and XLR may be one of them. Direct binding studies with ^{65}Zn are now underway.

(b) Another possible explanation for this "zinc-effect" is that some protein or structure in the nucleus depend on zinc ions for their stability and that it is the dissociation of zinc ions from this structure or molecule that causes the exit of XLR and other proteins from the nucleus, as previously de-

scribed for copper and calcium (Lebkowski and Laemmli, 1982a,b; Lewis and Laemmli, 1982).

Either of these explanations would be interesting with respect to mechanisms of nuclear localization. In addition, because the translocation of specific molecules from the cytoplasm to the nucleus is an important regulatory event in a number of systems, notably steroid hormone–receptor-induced cell differentiation (Yamamoto, 1985) and the movement of NF-κB in B cells (Sen and Baltimore, 1986), it seems possible that the zinc-dependent localization of XLR may have regulatory implications as well. Thus, changes in the intracellular free zinc ion concentration might control the location of XLR (and the related proteins seen in Fig. 7).

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