enzymes are most active at a relatively high pH (approximately 9.5). Although all four enzymes require Mg^{2+}, the optimum concentration for the KB_2 and calf thymus enzymes (5 mM) was greater than observed for either the KB_1 or EUE enzymes (0.5 and 1 mM). Furthermore, except for the KB_1 enzyme, all the other enzymes can utilize Mn^{2+} in place of Mg^{2+}. Additional distinctions among these enzymes are indicated by their different sedimentation coefficients and differences in relative rates of hydrolysis with poly(dT) as compared to denatured DNA. With regard to this latter feature, the two KB enzymes exhibited an intermediate ratio [about 7- to 8-fold greater degradation with poly(dT) as substrate] whereas the EUE enzyme had the highest ratio (15-fold) and the calf thymus enzyme the lowest degradation ratio (5-fold). Also, as shown in Table II, the relative rates of hydrolysis of homopolymers are clearly different among the calf thymus, KB_1, and KB_2 enzymes. A similar comparison was not included in the EUE enzyme study (Pedrini et al., 1976). Finally, it should be noted that there are two properties of the KB_2 enzyme that particularly distinguish it from the other three endonucleases: (i) degradation of linear double-stranded DNA does not occur with KB_2 endonuclease when KCl is omitted from the standard assay mixture whereas it does occur under similar conditions with each of the other enzymes and (ii) degradation of poly(dT) by the KB_2 enzyme is not inhibited by an increased salt concentration (up to 40 mM KCl), but similar increases in salt concentration markedly inhibit the degradation of poly(dT) by all the other enzymes.

Further questions relevant to KB_2 endonuclease specificity can be raised on the basis of several observations. First, alkaline sucrose gradient patterns suggest that the enzyme may have randomly nicked the single-stranded circular fd virus genome, yielding linear genome length molecules (Figure 3E,F; major slower sedimenting component). Alternatively, however, this cleavage could have been relatively site specific, a feature that might prove useful in the fractionation of certain single-stranded DNA molecules. An analysis of limit digestion products will be required to evaluate these possibilities. Secondly, unlike KB_1 endonuclease whose molecules appear to degrade both poly(dT) and poly(U) (Wang et al., 1978), KB_2 endonuclease can be readily purified away from any poly(U) degrading activity. Perhaps this difference relates to a difference in functions carried out by the two enzymes. Additional assessment to determine whether KB_2 endonuclease possesses any ribonucleolytic activity may thus be worthwhile.

In addition to the KB_1 and KB_2 endonucleases, we have observed that KB cells contain other endonucleases that hydrolyze poly(dT) more rapidly than denatured DNA. While little can be said at present concerning the specific cellular role of any of the endonucleases, there is a possibility that one or more of these enzymes may be involved in the replication of viral DNA (Wang et al., 1978).

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Inhibition of Double-Stranded Ribonucleic Acid Activated Protein Kinase and 2′,5′-Oligo(adenylic acid) Polymerase by Ethidium Bromide†

Corrado Baglioni* and Patricia A. Maroney

ABSTRACT: The activation of two enzymes induced by interferon, a protein kinase and the 2′,5′-oligo(adenylic acid) polymerase [2′,5′-oligo(A) polymerase], is inhibited by ethidium bromide. The activation of these enzymes requires double-stranded RNA (dsRNA), and binding of ethidium to dsRNA inhibits the activation process. This was shown by determining the concentration of ethidium inhibitory for poly(A)·poly(U)- and poly(I)·poly(C)-activated reactions. Activation of both protein kinase and 2′,5′-oligo(A) polymerase is inhibited by much lower concentrations of ethidium with the former polymer as activator than with the latter polymer. Correspondingly, in the presence of magnesium, ethidium binds with much greater affinity to poly(A)·poly(U) than to poly(I)·poly(C). Synthesis of 2′,5′-oligo(A) with poly(A)·poly(U) as activator is arrested by adding low ethidium concentrations, but it is resumed upon addition of poly(I)·poly(C). Kinase activity, however, is not inhibited when ethidium is added after the activating dsRNA. This suggests that the kinase interacts with dsRNA in a manner different from the 2′,5′-oligo(A) polymerase interaction.

Two enzymatic activities are induced by interferon in mammalian and avian cells, an oligonucleotide polymerase and a protein kinase [see Baglioni (1979) for references]. these enzymes are present in an apparently inactive form in extracts of interferon-treated cells and are activated by double-stranded RNA (dsRNA). The polymerase converts ATP into a series of oligonucleotides characterized by 2′,5′-phosphodiester bonds (Kerr & Brown, 1978) and designated 2′,5′-oligoadenylic acid [2′,5′-oligo(A)]. These oligonucleotides in turn activate an endonuclease that degrades RNA (Baglioni et al., 1978; Clemens & Williams, 1978). The protein kinase phosphorylates two polypeptides of M_r 38 000 and about 70 000, re-

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spectively [see Baglioni (1979)]. The first is the α subunit of initiation factor eIF-2; the latter polypeptide copurifies with the kinase induced by interferon (Kimchi et al., 1979).

Activation of the interferon-induced enzymes in virus-infected cells may play an important role among the antiviral defense mechanisms that inhibit the replication of a variety of viruses (Nilsen & Baglioni, 1979). Previous studies have investigated the size requirement of dsRNA for the activation of these enzymes (Minks et al., 1979b). Only dsRNAs longer than about 60–80 base pairs were fully active whereas dsRNA shorter than 35 base pairs and double-stranded polymers containing either a 2′-deoxy or a 2′-O-methylated strand were inactive (Minks et al., 1979a, 1980b). To further study the activation of the 2′,5′-oligo(A) polymerase and protein kinase, we have used ethidium bromide as an inhibitor. This compound intercalates between adjacent base pairs of dsRNA (Waring, 1965), causing an unwinding and a local extension of the duplex structure.

Ethidium inhibits both enzymes at concentrations characteristically dependent on the base composition of the homopolymeric dsRNA activating these enzymes. This has been explained by the intercalation of ethidium into dsRNA in the presence of physiological magnesium concentrations. Possible applications of these findings to the study of protein–nucleic acid interactions are discussed.

Experimental Procedures

2′,5′-Oligo(A) Polymerase and Protein Kinase Assay. High molecular weight ($s_{20,w}$ = 9–11 S) poly(I)·poly(C) and poly(A)·poly(U) were purchased from P-L Biochemicals; [^{3}H]ATP was from New England Nuclear. Extracts were prepared from HeLa cells treated for 17 h with 100 units/mL human fibroblast interferon as previously described by Minks et al. (1979a). Synthesis of 2′,5′-oligo(A) was assayed in 25-μL aliquots of reactions containing 5 μL of cell extract, 5 μg/mL dsRNA, 5 mM[^{3}H]ATP (about 200 000 cpm), 25 mM Mg(OAc)$_2$, 0.12 M KOAc, 4 mM fructose 1,6-bisphosphate, and 20 mM Hepes–KOH [Hepes, *N*-2-(hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid] (pH 7.4) (Minks et al., 1979a). The 2′,5′-oligo(A) synthesized was separated by chromatography on DEAE-cellulose as described by Minks et al. (1979a). Protein kinase activity was assayed by incubating for 7 min the ribosomal fraction obtained from interferon-treated HeLa cells with 0.5 μg/mL dsRNA and 4 μCi of [γ-^{32}P]ATP as described by West & Baglioni (1979). The phosphorylated proteins were resolved by gel electrophoresis, and autoradiographs were prepared and scanned in a recording spectrophotometer at 560 nm to measure the phosphorylation of a M_r 70 000 polypeptide (West & Baglioni, 1979).

Binding of Ethidium to Double-Stranded RNA. The binding of ethidium to dsRNA was measured spectrophotometrically by recording the absorption of 20 μM ethidium solutions to which dsRNA was added in variable amounts up to 2.5 mg/mL. Details of this procedure are given in the legend of Figure 4. Binding of ethidium was also measured by the fluorescence enhancement method, following the procedure described by Morgan et al. (1979). The ionic composition of the assays is described in the legend of Figure 5.

Results

The 2′,5′-oligo(A) polymerase activity was assayed in extracts of interferon-treated HeLa cells by measuring the conversion of [^{3}H]ATP into 2′,5′-oligo(A) as described by Minks et al. (1979a). The dsRNA-dependent protein kinase activity induced by interferon was quantitated by measuring the phosphorylation of a ribosome-associated polypeptide of

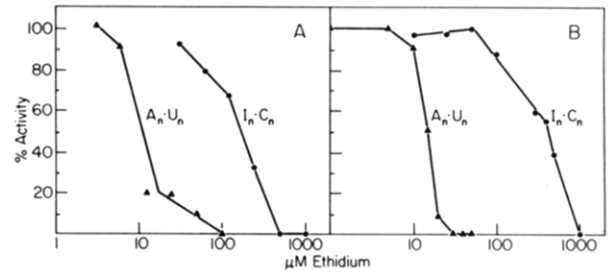

FIGURE 1: Inhibition of dsRNA-dependent protein kinase (A) and 2′,5′-oligo(A) polymerase (B) by ethidium bromide. The enzymatic assays are described under Experimental Procedures. (A) Phosphorylation of the M_r 70 000 polypeptide was measured in incubations containing 0.5 μg/mL of the dsRNA species indicated. The reactions were analyzed by gel electrophoresis (see Figure 2) and the gel autoradiogrpahs scanned. The kinase activity is expressed as a percent of that of incubations without added ethidium. (B) Synthesis of 2′,5′-oligo(A) was measured in 75-min incubations containing the ethidium concentration indicated on the abscissa and 5 μg/mL of dsRNA. With no added ethidium, an average of 17 nmol of ATP was converted to 2′,5′-oligo(A) from an input of 125 nmol; the relative synthesis in reactions containing ethidium is expressed as a percent of this value.

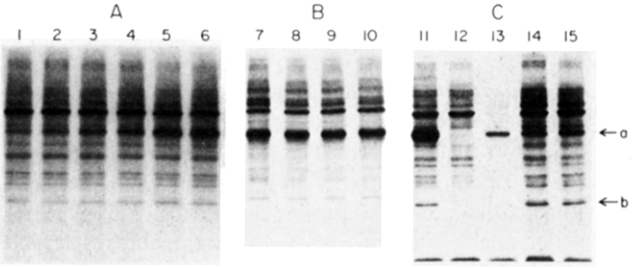

FIGURE 2: Inhibition of dsRNA-dependent protein kinase activity by ethidium bromide. (A) inhibition by ethidium added together with $A_n{\cdot}U_n$; ethidium concentrations (μM) are 50, 25, 12.5, 6.25, 3.125, and 0 in tracks 1–6. (B) Lack of inhibition when $A_n{\cdot}U_n$ is added 2 min before [γ-^{32}P]ATP and 0.1, 0.2, or 0.5 mM ethidium (tracks 7–9); control with no added ethidium (track 10). (C) Effect of ethidium on the phosphorylation of the M_r 70 000 polypeptide (a) and of eIF-2 (b). Reactions containing $A_n{\cdot}U_n$ and either no ethidium (track 11) or 0.1 mM ethidium (track 12) were incubated 7 min in a standard assay. The other reactions were first incubated for 15 min with unlabeled ATP and then for 7 min with 2 μCi of [γ-^{32}P]ATP and 0.1 mM ethidium (track 13). To two reactions was added an additional 40 μg of ribosomes from control (track 14) or interferon-treated cells (track 15) after the first incubation.

$M_r \simeq$ 70 000 according to West & Baglioni (1979). Addition of ethidium to assays of these enzymatic activities resulted in a dose-dependent inhibition (Figures 1 and 2A). Both activities were inhibited by low ethidium concentrations when poly(A)·poly(U) was the activator whereas they were inhibited by 50-fold greater concentrations when poly(I)·poly(C) was the activator (Figure 1).

This effect of ethidium may be explained by its interaction with the dsRNA required for the activation of the 2′,5′-oligo(A) polymerase and protein kinase. Intercalation of ethidium into dsRNA may change its structure in such a way that it no longer functions as an activator. To prove this, we showed that ethidium does not interfere with the catalytic function of the enzymes assayed but that it only prevents their activation. In subsequent experiments, we investigated the reasons for the widely different concentrations of ethidium required to inhibit enzyme activation by dsRNA and showed that this inhibition is directly related to the intercalation of ethidium into dsRNA.

Effect of Ethidium on 2′,5′-Oligo(A) Synthesis. Continuous presence of dsRNA is required for synthesis of 2′,5′-oligo(A), since digestion of dsRNA with RNase III stops the reaction

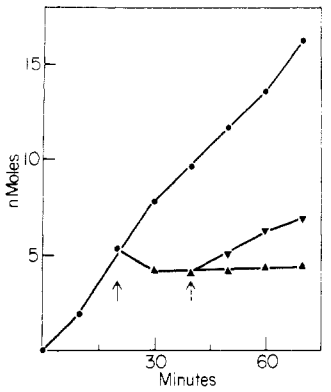

FIGURE 3: Reversible inhibition of 2′,5′-oligo(A) synthesis. A 0.5-mL reaction containing 5 μg/mL A_n·U_n and the other components described under Experimental Procedures was incubated at 30 °C; 25-μL samples were removed to measure nmol of ATP converted into 2′,5′-oligo(A) (●). After 20 min, 50 μM ethidium was added to a 0.25-mL aliquot (solid arrow); synthesis of 2′,5′-oligo(A) was assayed as described above (▲). After 40 min, 0.5 μg of I_n·C_n was added to a 0.1-mL aliquot of this incubation (broken arrow), and 2′,5′-oligo(A) synthesis was measured (▼).

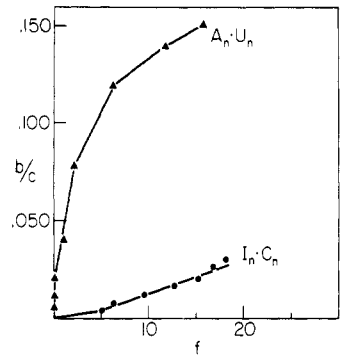

FIGURE 4: Binding of ethidium bromide to dsRNA. Solutions of 5 mg/mL of the indicated dsRNA were serially diluted 2-fold with ethidium-contg. buffer to a final concentration of 50 mM KCl, 10 mM $Mg(OAc)_2$, 20 mM Hepes–KOH, pH 7.4, and 20 μM ethidium. The samples were analyzed in a recording spectrophotometer, and the absorption at 450 nm was used to determine the relative amount of free ethidium indicated on the abscissa (f). On the ordinate is indicated the ratio (b/c) between bound ethidium and base pairs of dsRNA expressed as μM concentrations.

(Minks et al., 1980a). To show that ethidium inhibits the activation of the 2′,5′-oligo(A) polymerase, we took advantage of this observation and of the differential inhibition with the two synthetic dsRNAs shown in Figure 1. We followed the time course of 2′,5′-oligo(A) synthesis in a reaction containing poly(A)·poly(U) (Figure 3). The reaction proceeded linearly for at least 1 h, but addition of 50 μM ethidium to an aliquot of the incubation prevented further synthesis of 2′,5′-oligo(A). Interaction of ethidium with dsRNA is presumably responsible for abolishing the 2′,5′-oligo(A) polymerase activity, but this enzyme remains fully functional. This was shown by adding poly(I)·poly(C) to the reaction; synthesis of 2′,5′-oligo(A) resumed at the expected rate (Figure 3) since activation of 2′,5′-oligo(A) polymerase by poly(I)·poly(C) is only marginally inhibited by 50 μM ethidium (Figure 1). Therefore, this enzyme remains potentially active in the presence of ethidium and can apparently dissociate from poly(A)·poly(U) to interact with poly(I)·poly(C).

Effect of Ethidium on dsRNA-Dependent Protein Kinase. Experiments similar to the ones described above for the 2′,5′-oligo(A) polymerase were carried out to investigate the effect of ethidium on the activation of the interferon-induced protein kinase. When ethidium was added together with poly(A)·poly(U) before starting the reaction, a dose-dependent inhibition of phosphorylation was observed (Figure 2A); addition of poly(I)·poly(C) after 2 min of incubation restored the kinase activity (data not shown). When the reaction was allowed to proceed for 2 min before the addition of ethidium and [γ-^{32}P]ATP, however, the phosphorylation of the M_r 70 000 polypeptide was no longer inhibited (Figure 2B). The same result was obtained with ethidium concentrations up to 0.5 mM, concentrations that are the least 50-fold greater than those required to inhibit the activation of the kinase when ethidium is added together with poly(A)·poly(U).

These results suggest that once the kinase interacts with dsRNA it is no longer inhibited by ethidium. The phosphorylation of both kinase substrates, the M_r 70 000 polypeptide and the α subunit of eIF-2, is inhibited by ethidium (Figure 2C, track 12). When a reaction is preincubated for 15 min with unlabeled ATP before addition of [γ-^{32}P]ATP and ethidium, most proteins are phosphorylated during the preincubation, and no radioactive band is seen with the exception of a much reduced M_r 70 000 band (track 13). When a further aliquot of ribosomes from either control (track 14) or interferon-treated cells (track 15) is added to reactions preincubated in the same way, no increased phosphorylation of the M_r 70 000 polypeptide is observed whereas eIF-2α is phosphorylated. The kinase activated before ethidium addition apparently phosphorylates eIF-2α but not the M_r 70 000 polypeptide added with ribosomes of interferon-treated cells. A similar observation has been reported by West (1979).

Interaction of Ethidium with Poly(A)·Poly(U) and Poly(I)·Poly(C). The dsRNA-dependent enzymes studied are inhibited at lower ethidium concentrations with poly(A)·poly(U) as an activator than with poly(I)·poly(C). A rather high binding constant of ethidium for poly(A)·poly(U) at low ionic strength has indeed been reported by Baguley & Falkenhaug (1978), but there is no information on the binding of ethidium to different synthetic polyribonucleotides at the relatively high ionic strength of our assays and in the presence of magnesium. This cation was added at 20 mM concentration for the polymerase and at 2 mM for the kinase assay. Therefore, we measured the binding of ethidium to poly(A)·poly(U) and poly(I)·poly(C) under ionic conditions close to those of our enzymatic assays.

Initially, we measured this binding by adding decreasing amounts of dsRNA to a standard ethidium solution. This resulted in a spectral shift from the peak absorption of free ethidium at 480 nm to that of bound ethidium at 518 nm. The relative proportion of free and bound dye was calculated from the absorption at selected wavelengths and is shown in Figure 4. The ratio of bound ethidium per base pair of dsRNA (b/c) approaches saturation at a concentration of free ethidium (f) close to 20 μM with poly(A)·poly(U) whereas it is far from saturation with poly(I)·poly(C). This clearly indicated that ethidium binds with greater affinity to poly(A)·poly(U) than to poly(I)·poly(C).

To establish whether the binding of ethidium was due to intercalation of the dye, we measured the fluorescence enhancement described by Le Pecq & Paoletti (1967). Intercalation of ethidium in the hydrophobic environment of nuclei acid duplexes results in a large fluorescence enhancement, that can be assayed rapidly and sensitively according to Morgan et al. (1979). We measured the fluorescence of ethidium–dsRNA solutions in the absence of magnesium as described by Morgan et al. (1979) for assays at pH 8 and could not detect any significant difference between the synthetic dsRNAs studied (results not shown). In subsequent experiments, therefore, we measured the fluorescence enhancement at the

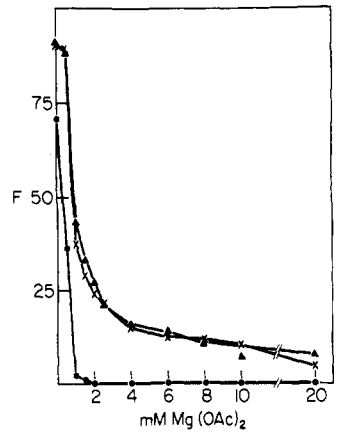

FIGURE 5: Effect of magnesium concentration on the fluorescence enhancement by ethdium bromide. The fluorescence at 600 nm of solutions containing 0.125 μM ethidium, 0.5 mM EDTA, 5 mM Tris-HCl, pH 7.4, and 0.25 μg/mL human placental DNA (×), A_n·U_n (▲) or I_n·C_n (●), was determined as a function of $Mg(OAc)_2$ concentration with an exciting light of 525 nm. The fluorescence scale is arbitrary; the sensitivity of the spectrofluorometer was adjusted with the DNA solution without added $Mg(OAc)_2$ to read 90%. The fluorescence of an ethidium solution without added nucleic acid (10%) was taken as background and subtracted from that of the samples analyzed.

pH and magnesium concentrations of the enzymatic assays and observed a marked decrease in fluorescence with the addition of magnesium. Interestingly, a systematic study of the effect of magnesium concentration on the fluorescence enhancement of DNA and of the two synthetic dsRNAs showed no fluorescence enhancement with poly(I)·poly(C) at magnesium concentrations higher than 2 mM (Figure 5). Both DNA and poly(A)·poly(U) showed decreased but significant fluorescence enhancement at magnesium concentrations up to 20 mM. This indicates that magnesium competes with ethidium for binding to poly(I)·poly(C) more effectively than for binding to the other nucleic acids. Other explanations for the effect of magnesium are also possible, however.

Discussion

The activation of protein kinase and 2′,5′-oligo(A) polymerase is inhibited by ethidium in a characteristic way. The initial inhibition of the kinase is observed at lower ethidium concentrations than those inhibitory for the polymerase, presumably because Mg^{2+} and dsRNA are present in the kinase assay at 10-fold lower concentration than in the assay for 2′,5′-oligo(A) synthesis. With poly(A)·poly(U) as activator, the inhibition shows a very sharp dependency on ethidium concentration for the 2′,5′-oligo(A) polymerase but a less steep inhibition curve for the kinase (Figure 1). We have calculated from the data of Figure 4 that complete inhibition of the polymerase is obtained when a molecule of ethidium is bound on average every eight base pairs of dsRNA. With poly(I)·poly(C) as an activator, ethidium is much less inhibitory because it binds poorly to this dsRNA in the presence of magnesium, as shown by the measurement of fluorescence enhancement (Figure 5).

This observation allowed us to establish that ethidium does not directly inhibit the protein kinase and the 2′,5′-oligo(A) polymerase activity, since the inhibition with poly(A)·poly(U) as activator can be reversed by the addition of poly(I)·poly(C). In the experiments shown in Figure 3, the synthesis of 2′,5′-oligo(A) was arrested by ethidium addition. When similar experiments were carried out with the kinase, however, ethidium added after the activating dsRNA was no longer inhibitory. This suggests that the two enzymes interact with dsRNA in different ways. The 2′,5′-oligo(A) polymerase presumably dissociates from poly(A)·poly(U) in the presence of ethidium whereas the kinase remains associated with poly(A)·poly(U) when this dsRNA is added before ethidium. The observation of Hovanessian & Kerr (1978) that the kinase is eluted from poly(I)·poly(C)–agarose at much higher salt concentrations than the polymerases is in agreement with this interpreation of our results.

The M_r 70000 polypeptide is apparently phosphorylated only when associated with an activating dsRNA (Figure 2C). This result is consistent with an observation of Farrell et al. (1977). A M_r 67000 polypeptide of rabbit reticulocytes (corresponding to the M_r 70000 polypeptide of HeLa cells; Lenz & Baglioni, 1978) is no longer phosphorylated by the dsRNA-dependent protein kinase when the dsRNA concentration is raised above a critical level whereas eIF-2 is still phosphorylated by the preactivated kinase. The reasons for this effect of dsRNA concentration on the pattern of phosphorylation of the kinase are unknown. In our experiments, an initial activation of the kinase may occur when dsRNA is added first, but further binding of the M_r 70000 polypeptide is prevented by the subsequent addition of the intercalating agent. Alternative explanations of this phenomenon are possible, however, in view of the recent report of Epstein et al. (1980) that poly(I)·poly(C) inhibits a phosphatase that specifically dephosphorylates a M_r 67000 polypeptide of L cells. This polypeptide (or the corresponding M_r 70000 polypeptide of HeLa cells) may be rapidly dephosphorylated unless bound to dsRNA. Intercalation of ethidium may prevent binding of this polypeptide to dsRNA and allow it to be dephosphorylated.

The observation that ethidium at physiological magnesium concentrations intercalates preferentially into poly(A)·poly(U) may be of general interest. The intercalating agent proflavin inhibits the cleavage of SV-40 nuclear RNA precursors of mRNA (Chiu et al., 1979). This observation has suggested that base-paired sequences of primary transcripts are important processing signals in the synthesis of mRNA (Chiu et al., 1979). Similarly, treatment of mouse kidney cells in culture with ethidium inhibits ribosome biosynthesis, with formation of 28S rRNA preferentially inhibited over that of 18S rRNA (Lange & May, 1979). Since 28S rRNA does not accumulate in the nucleus, ethidium affects either its stability or, more likely, its processing from the 32S precursor (Lange & May, 1979). These results suggest that ethidium may be used as a probe for determing whether base-paired sequences of RNA are A·U rich; at some critical concentration, ethidium would alter the secondary structure of these sequences and inhibit processing enzymes that may interact with dsRNA. Indeed, ethidium intercalation prevents the degradation of dsRNA by a nuclease activity of mammalian cell nuclei (Saha & Schlessinger, 1978). An analysis of nucleotide sequences involved in processing signals may provide direct support for the hypothesis that ethidium interacts preferentially in intact cells with A·U-rich dsRNA.

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Cloning and Characterization of a Highly Reiterated 5.8-Kilobase Pair Nucleolar *Eco*RI DNA Fragment Found in Novikoff Hepatoma Ascites Cells†

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ABSTRACT: The DNA of Novikoff hepatoma ascites cells was found to contain a 3.6-megadalton *Eco*RI restriction fragment, referred to as *Eco*RI fragment A (Parker et al., 1979). C_0t analyses demonstrated an enrichment of fragment A sequences in Novikoff hepatoma genome relative to normal rat liver DNA. This fragment was cloned in λ gtWES to determine its molecular structure and sequence organization. The DNA from a positive clone was labeled by nick translation and hybridized to a Southern blot of *Eco*RI digested Novikoff DNA. Distinct hybrids formed with the region corresponding to fragment A. The greater degree of hybridization to the nucleolar fraction suggested a nucleolar enrichment of fragment A. Fragment A has a *Pst*I site approximately 300 base pairs from one terminus which was used to generate mono-5′-^{32}P-labeled fragments. The larger *Pst*I subfragment, 5500 base pairs, labeled at a single terminus, was used to evolve a restriction enzyme map. The 300 base pair fragment was partially sequenced, revealing the presence of a repetitive sequence "island", $TT(GTCT)_8(GAAT)_5G$-. C_0t analysis, utilizing the purified clone as a probe, confirmed the enrichment of fragment A sequences in the tumor relative to the normal rat liver control.

When DNA prepared from the nucleoli of Novikoff hepatoma ascites cells was digested with any of a number of restriction endonucleases and subjected to agarose gel electrophoresis, various dense ethidium bromide staining bands were found which ranged in size from approximately 1000 base pairs to 7500 base pairs (Parker et al., 1979; Fuke & Busch, 1979). When similar digestions were carried out with whole nuclear DNA prepared from Novikoff hepatoma cells, a distinct subset of these bands was not seen. When the DNA of normal rat liver nuclei or nucleoli was digested similarly, this subset of bands was not found (Parker et al., 1979; unpublished observations).

Digestion of Novikoff nucleolar DNA with the restriction endonuclease *Eco*RI generated three ethidium bromide staining bands (in the molecular weight range greater than 1000 base pairs) (Parker et al., 1979). These fragments, referred to as fragments A, B, and C, contained 5800, 2100, and 1400 base pairs, respectively. Fragment A was not found in similar digests of either normal rat liver nuclear or nucleolar DNA whereas fragments B and C were seen. Comparative *Eco*RI digestions of Novikoff hepatoma nucleolar and nuclear DNA suggested that fragment A was localized predominately in the nucleolus. Nucleoli prepared by several methods (Busch & Smetana, 1970) all contained relatively similar amounts of fragment A. Previous hybridization studies, utilizing a radiolabeled fragment A probe, demonstrated that fragment A was amplified in the Novikoff hepatoma cells in comparison to normal rat liver cells. Fragment A comprised approximately 5% of nucleolar DNA and did not cross-hybridize with rRNA or any known rDNA fragments. Its reiteration number, approximately 6000, as well as its large size suggested that it was not part of the rDNA repeats which number 250–500 (Parker et al., 1979).

In the present study, we report on the successful cloning of the *Eco*RI fragment A in λ gtWES. Using this clone as a hybridization probe we were able to confirm a nucleolar

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